Lentiviral vector vaccines for T cell-mediated protection against influenza

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Declaration

I, Douglas Christian Macdonald, confirm the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Vaccines that induce T cells which recognize conserved viral proteins could confer cross-strain protection against pathogens with fast-mutating B cell epitopes. Influenza is an example of such a pathogen for which there is a pressing need for a universal vaccine. Lentiviral vectors are a counterintuitive choice as vaccines since they have low inherent immunogenicity. However, their efficient transduction of non-dividing cells and high capacity permits transduction of antigen presenting cells with not only antigen but also molecular adjuvants that directly or indirectly enhance the T cell response. We therefore investigated the potential of two such adjuvants: viral flice-like inhibitor protein, which activates dendritic cells through nuclear factor kappa-B, and 4-1BB ligand, which activates T cells directly through 4-1BB. By co-encoding these with influenza nucleoprotein, we have shown that the influenza-specific T cell response to lentiviral vector vaccination is significantly enhanced in mice. Furthermore, we have demonstrated that intranasally delivered lentiviral vectors transduce alveolar macrophages with high efficiency, recalling and expanding large and sustained populations of nucleoprotein-specific CD8+ T cells in the lung and airway in mice that have been primed subcutaneously or previously exposed to influenza. These lung-resident T cell populations persist for at least 4 months and are sufficiently abundant to rapidly control a mouse-adapted lethal influenza challenge without invocation of a secondary cytokine response, weight loss or lung injury. Furthermore, dendritic cells expressing 4-1BBL potently trans-activate bystander dendritic cells, both *in vitro* and *in vivo*, demonstrating an indirect mechanism by which the 4-1BBL:4-1BB signaling axis can enhance T cell responses.

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A/Eng/195	A/England/195/2009
A/PR/8/34	A/Puerto Rico/8/1934
AAV	adeno-associated viruses
APC	antigen presenting cells
BCA	bicinchononic acid
BCN	broadly cross-neutralising
BAL	broncho-alveolar lavage
BALT	bronchus associated lymphoid tissue
CFSE	carboxyfluorescein succinimidyl ester
CEA	carcinoembryonic antigen
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
TCM	central memory T cells
cPPT	central polypurine tract
C1orf190	chromosome 1 open reading frame 190
CRM-1	chromosome region maintenance 1 protein
JNK	c-Jun protein kinase
CPSF	cleavage and polyadenylation specificity factor
CAIV	cold-adapted influenza viruses
cDNA	complementary DNA
CTL	cytotoxic T lymphocytes
DAMP	damage-associated molecular patterns
DCIR-2	dendritic cell immunoreceptor 2
DC	dendritic cells
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
dNTP	deoxynucleotide triphosphates
DNA	deoxyribonucleic acid
DLPC	dilauroylphosphatidylcholine
DTR	diptheria toxin receptor
DsDNA	double stranded DNA
DMEM	Dulbecco's Modified Eagle's Medium
TEM	effector memory T cells
EB	elution Buffer
ECL	enhanced chemiluminescence
ELIspot	enzyme-linked immunospot assay
ERK	extracellular signal regulated kinases
FasL	Fas ligand
FACS	fluorescence-activated cell sorting
gp120	glycoprotein 120

GVHD	graft versus host disease
GMCSF	granulocyte monocyte colony stimulating factor
GzmB	granzymeB
GFP	green fluorescent protein
GAG	group antigens
HA	haemagluttinin
HBSS	Hank's balanced salt solution
HSP	heat-shock protein
HBc	hepaitis B core
HBV	hepatitis B virus
HSV	herpes simplex virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
Ig	immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motifs
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
IRES	internal ribosomal entry site
ICCS	Intracelluar cytokine staining
IN	intranasal
KSHV	Kaposi's sarcoma herpes virus
LV	lentiviral vectors
LUBAC	linear ubiquitination associated complex
LPS	lipopolysaccharide
LAIV	live attenuated influenza vaccination
LTR	long terminal repeats
LB	Luria Bertani agar
LN DC	lymph node-resident DC
LFA-1	lymphocyte function-associated antigen 1
LCMV	lymphocytic choriomeningitis virus
MIP	macrophage inflammatory protein
MDCK	Madin-Derby canine kidney
MHC	major histocompatibility complex
M1	matrix protein 1
MDA	melanoma differentiation associated factor
MAPK	mitogen-activated protein kinase
MVA	modified vaccinia Ankara
MCP	monocyte chemoattractant protein
MyD88	myeloid differentiation factor 88
NALT	nasal associated lymphoid tissue
NK	natural killer

NA	neuraminidase
NEMO	NFkB essential modulator
NIK	NFκB inducing kinase
NI	non-integrating
NSP	non-structural protein
NFκB	nuclear factor kappaB
NP	nucleoprotein
OVA	ovalbumin
PRR	pathogen recognition receptors
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PGK	phosphoglycerate kinase promoter
PABPII	polyA binding protein II
PAP	polymerase acidic protein
PB1	polymerase basic 1
PCR	Polymerase chain reaction
PDL-1	programmed cell death ligand-1
PKR	protein kinase R
PAP	pulmonary alveolar proteinosis
RAF-1	rapidly accelerated fibrosarcoma-1
RANKL	receptor activator of NFκB ligand
RANTES	regulated on activation, normal T cell expressed and secreted
RSV	respiratory syncytial virus
RIG	retinoic acid inducible gene
RLRs	retinoid acid-inducible gene (RIG)-I-like receptors
RRE	rev response element
RT	reverse transcriptase
RNA	ribonucleic acid
RNP	ribonucleoprotein
RdRp	RNA dependent RNA polymerase
RPMI	Roswell Park Memorial Institute
SCID	severe combined immunodeficiency
SIV	simian immunodeficiency virus
SAMHD1	sterile alpha motif domain and HD domain containing protein
SC	subcutaneous
SPD	surfactant protein D
SOH1N1	swine-origin H1N1
TCR	T cell receptor
TH	T helper
T reg	T regulatory
TBK-1	TANK-binding kinase -1

TRAF	TNF-receptor associated factor
TIR	toll/IL-1 receptor
TLR	toll-like receptor
TFB	transformation buffer
TGF-β	transforming growth factor- β
TAE	tris-acetate EDTA buffer
TE	Tris-EDTA
TB	tuberculosis
TNF	tumour necrosis factor
Tpl2	tumour progression locus 2
TRP2	tyrosinase-related protein-2
URT	upper respiratory tract
VV	vaccina virus
VZV	varicella zoster virus
VSV	vesicular stomatitis virus
vFLIP	viral flice-like inhibitor protein
WT	wild-type
WPRE	woodchuck hepatitis virus posttranscriptional regulatory element

1 Introduction

1.1 An overview of vaccination for T cell mediated immunity

No other field of medicine has had a greater impact on human disease than vaccination. When smallpox was eradicated in 1979, it was estimated to have killed between 300-500 million people worldwide in the 20th century alone. A greater than 99% decline in deaths due to diphtheria, mumps, pertussis and tetanus has been seen in the US since the introduction of national vaccination programmes for these diseases[1]. These vaccines were developed long before a detailed understanding emerged of the immune mechanisms that underlie their efficacy, such as antigen-presenting cell maturation, T cell co-stimulation, T cell help and B-cell activation. They were also predominantly antibody-based strategies, wherein clinical efficacy relied upon maximizing titres of neutralising antibody against external pathogen epitopes. This approach has been of limited success against pathogens with rapidly mutating antigenic profiles, multiple redundant mechanisms of infection, several circulating serological strains or a life cycle that occurs in intracellular compartments to which antibodies have no access. These are unfortunately characteristics of some of the most prolific infectious diseases of our time, such as malaria, tuberculosis (TB), human immunodeficiency virus (HIV), viral hepatitis and influenza.

The last of these presents particular challenges for the design of effective countermeasures since it combines rapid replication, high infectivity by aerosol spread, a fast mutation rate and the ability to reassort in hosts co-infected with different strains. Prior to emergence of the 2009 swine-origin H1N1 (SOH1N1) influenza strain, pandemic influenza was rated by the National Risk Register agency as the highest threat to the UK population as a combined function of likelihood and impact. SOH1N1 turned out to be a relatively mild strain but its rapid global spread highlighted the inadequacy of current vaccination technology for pandemic control. Furthermore, despite annual vaccination programmes, the impact of all the previous pandemics combined is dwarfed by the

cumulative morbidity and mortality from seasonal influenza. Influenza is estimated to contribute the deaths of some 36,000 people in the US per year, whilst the WHO estimate annual worldwide mortality figures are between 250,000 and 500,000[2].

Antibody-based vaccination strategies are the cornerstone of influenza countermeasures. However, the rapid mutation rate of haemagluttinin (HA) and neuraminidase (NA) B-cell epitopes on the influenza viral surface presents a major problem for vaccine design. Trivalent seasonal vaccines in current use require annual updating to incorporate subtypes and strains of the previous season (currently H3N2, H1N1 and Influenza B). These are inevitably out of step with circulating subtypic variants.

Generating T cell responses against conserved and vital elements of pathogens that otherwise mutate rapidly or exist in several strains is a promising strategy to prevent or treat such diseases. Traditional protein and adjuvant-based vaccines, however, tend to generate very weak T cell responses. The trivalent inactivated seasonal influenza vaccine, for example, generates poor CD4+ or CD8+ T cell responses in children and adults[3] or mice[4]. Indeed, repeated vaccination of children against influenza with seasonal vaccines may inhibit development of T cell mediated heterosubtypic memory which may otherwise be boosted through natural infection[5,6].

A number of vaccination approaches have been developed to enhance T cell responses to a chosen antigen. In broad terms, the aim of these strategies is to maximize antigen presentation by antigen presenting cells (APCs) to T cells whilst also ensuring delivery of sufficient co-stimulation to ensure appropriate T cell activation. Dendritic cells (DC), macrophages and B cells are all capable of uptake of antigen or immune complexes via phagocytosis, endocytosis, pinocytosis and specific receptors for immune presentation. Extracellularly-acquired antigens are processed onto class II major histocompatibility complex (MHC) molecules, whereas class I MHC molecules present antigens synthesised within the cytosolic compartment. DC, however, have the capacity to cross-present exogenous antigen on class I MHC receptors. This, together with their high expression of co-stimulatory molecules and cytokines, makes them particularly efficient stimulators of effective T cell responses directed at intracellular pathogens. A number of successful

strategies have therefore emerged which harness the potency of DC in generating antigen-specific T cell responses:

i) **Ex-vivo generated DC-based vaccines.** Culturing, maturing and antigen-pulsing DC from human peripheral blood progenitors before re-administration has shown considerable clinical promise as a means of stimulating immunotherapeutic T cell responses against cancer and some chronic infections such as HIV.

ii) ***In vivo*** **DC targeting.** Formulations that maximise the uptake of incorporated antigen by DC after subcutaneous or intravenous administration increase T cells responses whilst avoiding the need for costly *in vitro* DC manipulation.

iii) **Live attenuated infection**. Attenuated live viruses may directly infect DC, ensuring efficient endogenous antigen processing and DC activation. In the absence of direct APC infection, replicating pathogens provide a considerable volume of antigen for cross presentation and stimulation of pathogen recognition receptors for DC activation (see below).

iv) **DNA and viral vector vaccines**. Genetic modification of APCs through use of DNA- or viral vector-based vaccines generate potent T cell responses by combining endogenous expression and class I processing of antigen together with vector- or DNA - mediated stimulation of pattern recognition receptors (such as Toll-like receptors) ensuring DC activation and T cell co-stimulation.

These are discussed in turn below, with an emphasis on viral vector vaccination which is the principle subject of this thesis. However, it is noteworthy that for simplicity the efficacy of these approaches is often explained in the literature as enhancing a linear axis of immunity, beginning with DC uptake of antigen (or transduction/transfection) in the periphery after vaccination, migration to lymph nodes and then stimulation of effector CD8+ and CD4+ T cells by antigen presentation antigen (MHC class I and MHC class II

receptors respectively) together with co-stimulation through surface DC molecules and DC-secreted cytokines. However, this is an oversimplification of the complex interaction - both stimulatory and inhibitory - that occurs between DC, T cells, B cells and other innate and adaptive arms of the immune system. Whilst an antibody or T cell response may be the focused objective of a given vaccine approach, one is rarely generated without the other and these are often mutually enhancing. For example, antibodies targeted against an internal virion component may not be capable of neutralising a virus but may assist the T cell response by enhancing antigen uptake and cross presentation by DC to T cells. Similarly, CD4+ T helper (TH) cell responses are needed for efficient antibody responses, influencing both their magnitude and isotype. Just as there is no simple linear chain of immune responses to vaccination, communication between immune cells is neither exclusively unidirectional nor between heterologous cell types. Cross-activation and antigen transfer between afferent migrating DC (such as skin Langerhans cells) and lymph-node resident DC may be essential for amplification of antigen presentation and thus effective T cell priming, a concept explored further in Section 6.5. CD4+ T cell cytokine responses can feedback on DC and enhance their activation, or alternatively, where a regulatory T cell response has been induced, this can powerfully suppress DC activation as well as inhibiting the effector functions of CD8+ T cells.

This presents such complexity that to examine every arm of the immune response to prophylactic or therapeutic vaccination, and the interaction between each of these arms, is beyond the scope of most studies. There are historical examples of the perils of underestimating this complexity. Respiratory syncytial virus (RSV) vaccine trials in the 1960s testing a formalin-inactivated RSV found increased morbidity and mortality in vaccinated subjects following subsequent RSV infection. Despite generating the desired antibody response against RSV epitopes, vaccination had also primed an excessive CD4+ TH2 response which coordinated an deleterious eosinophilic influx on secondary challenge[7]. This example serves as a caveat to all vaccination work that intends to induce protection or therapy though a specific arm of the immune system, including that presented in this thesis.

1.1.1 Ex-vivo generated DC-based vaccines

The discovery of methods for culture, expansion and differentiation of human DC *in vitro* from peripheral blood CD14+ progenitors in the mid-1990s led to both rapid progress in understanding of human DC biology and also their use for immunotherapy[8]. This has principally been applied to generating therapeutic rather than preventative T cell responses against cancer or chronic infection, since the culture, modification and re-administration of autologous DC is prohibitively time-consuming and expensive for use in prophylactic vaccination of large populations. However, this approach does permit optimal "loading" of DC with an array of peptides or whole antigen and maturation of DC ensuring T cell responses are both multispecific and polyfunctional respectively.

Most of the development of this technique has occurred in the context of cancer immunotherapy and has befitted from key advances in understanding of DC and T cell biology. For example, it rapidly became apparent that DC must be activated to generate effective anti-tumour T cell responses. This discovery paralleled the emerging realisation that DC play a key role in the maintenance of tolerance to self and innocuous antigen. Immature DC can tolerise T cell responses against pulsed antigen (by deletion, anergy or generation of T suppressor cells) and mature DC can take on a "tolerogenic" phenotype and actively generate inducible T regulatory (T reg) cells through, for example, secretion of indoleamine 2,3-dioxygenase (which depletes tryptophan in T cells) and transforming growth factor-β (TGF-β) signalling[9]. Indeed, the recognition that inhibition or depletion of the regulatory immune system during DC-based immunotherapy greatly enhances T cell responses has been a further key step forward in this field.

Ex-vivo DC–based cancer vaccines have been used in clinical trials for almost two decades with mixed results in typically "immunosensitive" tumours such as melanoma, prostate cancer and lymphoma (reviewed in[10]). A recent notable success was the used of enriched blood APCs cultured with a fusion protein of prostatic acid phosphatase and granulocyte monocyte colony-stimulating factor (GM-CSF) which enhanced anti-tumour T cell responses and survival in metastatic prostate cancer by 4 months in Phase III trials[11].

Ex-vivo DC based therapies have also been applied in HIV infection. A recent clinical trial was based on successes in a simian immunodeficiency virus (SIV) model using monocyte-derived DC generated ex-vivo with GM-CSF and interleukin (IL)-4 matured with IL-1β, IL-6 and TNFα and loaded with inactivated, autologous HIV. Subcutaneous injection of these DC reduced median plasma HIV RNA levels by over 90% in almost 50% of subjects, correlating with the emergence of HIV-specific T cells in these subjects. Several other studies (reviewed in[12]) have shown promise using ex-vivo modified DC for HIV therapy, but since the demonstration in the Strategies for Management of Anti-Retroviral Therapy (SMART) study of a twofold risk of HIV progression or death in patients on intermittent rather than continuous therapy[13], to suspend therapy to observe the impact of any immunotherapeutic intervention is becoming increasingly questionable.

The obvious drawbacks of this approach for mass prophylactic vaccination against influenza explain why only one animal study has been performed assessing this approach, with a view to its use in immunosuppressed patients with particular vulnerability to influenza infection. However, this was focussed on using re-administered, ex-vivo pulsed DC for improving antibody rather than T cell responses to influenza which might be otherwise be attenuated in immunosuppressed individuals[14].

1.1.2 *In vivo* DC targeting

The expense and time involved in ex vivo modification of DC may be avoided by targeting DC *in vivo* with both antigen (such that it is efficiently taken up and cross-presented) and adjuvant (such that T cell co-stimulation is optimised). The current mainstay of targeting DC employs chimeric proteins composed of an anti-DC receptor antibody and antigen. This approach has been used to target distinct DC subsets with correspondingly different profiles of T cell activation. For example, ovalbumin (OVA) coupled to antibody targeting DEC-205+ DC preferentially induces CD8 + OVA-specific T cell immunity, whereas targeting dendritic cell immunoreceptor 2 (DCIR2) generated stronger CD4+ T cell responses. Chimeric antigens in which the antibody fragment targets activatory receptors on DC, such

as the lectins Dectin-1 or Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) can also modulate DC function. For example, DC-SIGN activates rapidly accelerated fibrosarcoma-1 (RAF1), which induces toll-like receptor (TLR)-independent phosphorylation of the nuclear factor kappa B (NFκB) subunit p65 which in turn up-regulates an array of DC co-stimulatory molecules that favour enhanced CD8+ T cell responses[15].

Another means of DC targeting exploits the existing physiological mechanism by which antibody-captured antigen can be internalised by DC for cross presentation. Fc receptors bind the Fc portion of antibodies and are expressed on different leukocyte cell types with specificities for specific antibody classes and subclasses. FcγR1 is constitutively expressed on DC and has a high affinity for monomeric immunoglobulin isotype G (IgG). It carries immunoreceptor tyrosine-based activation motifs (ITAMs), capable upon cross-linking by antibody-antigen complexes of initiation of both phagocytosis and also activatory signals that increase expression of DC co-stimulatory molecules and presentation of processed antigen[16]. By fusing antigen to monoclonal antibodies targeting Fc receptors or to the Fc domain of IgG, or by co-administering antigen with antigen-specific antibody, the Fc receptor uptake pathway can be exploited for improved antigen presentation and co-stimulation by DC.

HIV glycoprotein 120 (gp120), for example, elicits superior humoral and T cell responses in mice when fused to an anti-Fc monoclonal antibody[17]. A fusion product of influenza HA and the Fc domain of immunoglobulin has also been used to generate protective titres of anti-HA antibody and T cell responses in mice in the absence of adjuvant, and can be readily expressed in a baculovirus-insect cell system for scalability[18]. Similarly, incorporating an α–Gal epitope (which is abundant on glycolipids and glycoproteins of bacteria) into HA, exploits the ubiquitous and high level expression (over 1% of all immunoglobulin found in human serum) of anti-α–Gal antibody in humans (generated by antigenic stimulation by bacteria of the gastrointestinal flora). Inactivated A/Puerto Rico/8/1934 (A/PR/8/34) influenza virus engineered to express α–Gal epitopes generated much stronger CD4+ and CD8+ T cell responses against influenza epitopes than

unmodified inactivated virus[19]. However, it was not determined whether these T cell responses were sufficient to confer protection against a heterosubtypic influenza strain.

As with ex-vivo DC-based therapies, *in vivo* DC targeting has mostly been tested in therapeutic vaccination in mouse models of cancer. These approaches are capable of generating potent CD4+ and CD8+ anti-tumour immunity that is frequently partially protective against tumour challenge (typically in an OVA-expressing tumour challenge model, reviewed in[20]), but only In the presence of adjuvants to ensure effective DC activation. Only one study has directly compared *in vivo* DC-targeting with *ex vivo* antigen-loaded DC, finding that mice therapeutically vaccinated with anti-DEC-205-OVA and anti-CD40 mAb (as adjuvant) induced more potent OVA-B16 melanoma tumour inhibition than mice vaccinated with DC that had been OVA loaded and activated ex-vivo[21].

Unlike ex-vivo DC-based vaccination studies, few in-vivo DC targeting candidate vaccines have been tested in clinical trials. In primate studies, anti-DC SIGN mAb has been shown to target APC[22] and conjugation of HIV group antigens (GAG) p24 to anti-DEC-205 mAb resulted in better cross-priming of GAG-specific CD8+ T cells[23]. However, DEC-205 is not restricted to DC in humans to the same degree as mice, questioning the targeting specificity of this approach.

The requirement for adjuvant (and its implications for vaccine storage, production and safety) and the scarcity of truly DC-specific targets are not the only challenges in taking this vaccination strategy forward. Not all antigens are readily recombined with antibody in a manner that can be reliably secreted in yeast, bacterial or plant expression systems. Influenza HA is a case-in-point, a problem recently circumvented by use of a non-covalent system of binding recombinant DC-targeting antibody (anti-langerin) with HA by fusing each to dockerin and cohesin respectively – two *E. Coli* proteins that bind with high affinity[24]. This approach improved T cell responses against HA compared with vaccination with the antigen alone, but at the expense of significant manufacturing complexity.

Nanoparticles and liposomal vaccines may address these problems. These can encapsulate (within a glycoprotein or liposomal capsid respectively) both antigen and adjuvant and targeting mAb can be engineered to be expressed at the surface by recombination or covalent attachment. They have the further advantage of multivalent expression of targeting antibody, leading to better cross-linking of target receptors on DC and thus improved DC activation. Furthermore, the repetitive antigen structures of nanoparticles and liposomes can stimulate pathogen recognition receptors on DC, enhancing their activation and stimulating phagocytosis. Indeed, even in the absence of DC-targeting antibodies on their surface, nanoparticles and liposomes are avidly taken up by phagocytic cells including DC and macrophages[25]. Many of the current leading candidate vaccines for generation of mucosal T cells against influenza and other respiratory infections are nanoparticle formulations, which is at least in part attributable to their avidity for alveolar macrophages after intranasal administration (discussed further in Section 4.1.2).

However, the development of increasingly complex nanoparticle and liposomal technologies to mimic the potent DC activation, uptake and antigen presentation to T cells disregards an important strategic short-cut. The immune system has co-evolved with intracellular pathogens to reliably generate T cell memory after primary infection which confers protection against secondary challenge, albeit to varying degrees. It may be considerably more effective and simpler, therefore, to modify pathogens in such a way that they are rendered less harmful but more immunostimulatory, rather than engineer complex virus-like particulate agents with apparently desirable immunostimulatory characteristics from scratch.

1.1.3 Live attenuated vaccines

Many of the most successful vaccines against viral disease– polio, small pox, measles, mumps, rubella, yellow fever – have used replication competent attenuated forms of parent organisms. This is typically achieved by deletion of pathogenicity factors in the viral genome or passage through a non-human host until adaptations for virulence in humans are lost. Attenuated live organisms have the advantage instigating T cell responses similar to those against the parent organism in terms of multi-epitope specificity and functional phenotype. In nearly all cases, however, protection is mediated by high titres of neutralising antibody against surface viral epitopes, achieved with aid of accompanying CD4+ T helper cell responses which obviate the need for adjuvant or booster vaccination in many cases.

However, live attenuated vaccines tend to induce weaker T cell responses than infection with the wild type organism, since T cell responses are generally proportionate to antigen quantity and disease severity (discussed further in section 1.3). This may be sufficient to keep latent infection at bay by boosting an existing T cell memory population. This is demonstrated by live attenuated varicella zoster virus (VZV) vaccines for prevention of shingles in older adults previously exposed to chicken pox, wherein anti-VZV antibodies remain high but T cell mediated control of latent infection diminishes with age[26]. Nevertheless, to date there are no live attenuated vaccines in clinical use that mediate protection by a T cell predominant mechanism in the absence of neutralising antibodies.

Live attenuated influenza vaccination (LAIV) typically uses cold-adapted influenza viruses (CAIV) which replicate inefficiently at human body temperature. Naïve mice demonstrate heterosubtypic immunity after LAIV comparable to that seen after non-attenuated sub-lethal infection[27]. However, LAIV generates detectable T cell responses in children but does not enhance flu-specific peripheral T cell responses in adults with pre-existing cognate T cell memory[28]. Mirroring this finding, the clinical efficacy of CAIV is slightly superior to the standard inactivated trivalent vaccine in children but not adults[29]. Since the current licensed intranasal cold-adapted influenza vaccine (Flumist®) is seasonally

engineered to represent circulating strains and their HA and NA subtypes it is unclear to what degree the protection conferred is attributable to T cell mediated immunity rather than sterilising antibody responses. There are also safety concerns surrounding the use of an attenuated pathogen with a high mutation rate, which risks reversion to a body-temperature adapted phenotype. In one Phase III trial, LAIV compared with seasonal trivalent vaccination was associated with an increased incidence hospitalization in children aged 6-11 months and an increased incidence of clinical symptoms of bronchospasm in children <24 months. However, subsequent trials in children with asthma have not shown increased adverse events associated with LAIV vaccination compared with the inactivated trivalent seasonal vaccine[30].

1.1.4 DNA vaccines

Soon after the discovery that DNA plasmid injected into mouse model resulted in expression of encoded protein[31], it was realized DNA can also generate an antibody [32]and T cell response[33] to encoded antigen. Importantly, DNA vaccines have the advantage of being self-adjuvanting. This was initially attributed to inclusion of unmethylated CpG sequences present in certain plasmids that stimulate TLR-9 on APC activation, but it has recently become evident that these are not necessary for the induction of innate immunity[34]. Double stranded DNA (dsDNA) alone can activate APCs through TANK-binding kinase-1 (TBK-1, a non-canonical IkappaB kinase) pathways and is necessary and sufficient for the immunogenicity of DNA vaccines[35].

Pre-clinical studies of DNA vaccines in small animals focused primarily on antibody induction against targets such as herpes simplex virus (HSV), hepatitis B virus (HBV), HIV and influenza with consistent demonstrations of protective immunity[36–38]. The ability of DNA vaccines to generate cellular responses led to a proliferation of clinical trials of prophylactic and therapeutic DNA vaccines against cancers (including prostate[39], melanoma[40] and breast[41]) and infections (including HIV[42], malaria[43]and HBV[44]). Much of the early attempts to generate T cell mediated heterosubtypic protection against

influenza were achieved in mice using DNA vaccination (which are revisited in more detail in section 3.5).

In 2005, two DNA vaccines were authorized for veterinary use in the US: one against West Nile virus infection in horses, the other against canine melanoma. A further DNA vaccine has been licensed for use in Canada for the prevention of infectious haematopoeitc necrosis virus in salmon.

However, whilst human studies have consistently demonstrated the excellent tolerability and safety profiles of DNA vaccines, efficacy in generating T cell or antibody responses in humans and non-human primates is significantly lower than that seen in mice and other species. This may be a consequence of lower levels of APC transfection both *in vitro* and *in vivo* and thus reduced endogenous antigen expression and class I antigen processing by DC[45]. Much of the T cell response in humans to DNA vaccination must therefore be generated by APCs that have picked up exogenous antigen shed by other transfected cells or phagocytosed from dead cells, thus relying upon cross-presentation for class I presentation of antigen to cytotoxic T lymphocytes (CTL). This does not preclude the generation of effective T cell responses – a number of tissue-tropic viruses (e.g. pox viruses) do not infect APCs directly but nevertheless elicit T cell immunity via cross-presentation of viral antigens. However, viral infection (or viral vector vaccination) induce a number of signals not present after DNA vaccination. Apoptosis of antigen-bearing cells is sufficient to generate cross-presentation, inducing phagocytosis by immature DC, maturation and cross presentation[46]. This apoptosis has to be substantial, since the phagocytic capacity of scavenging macrophages (which secrete DC-inhibitory factors such as IL-10) must first be exhausted before less numerous DC are exposed to apoptosing cells[47]. Cross presentation is also significantly boosted by the presence of CD4+ T cell help and the presence of “danger signals” interacting with pathogen recognition receptors (PRR) and enhancing DC activation – both significantly more abundant following viral infection than DNA transfection of tissue cells. Indeed, secretion of antigen from transfected cells alone may not only be inadequate to induce a CTL response, but also may

risk generating “cross-tolerance” by cross-presentation of antigen in the absence of co-stimulation, leading to deletion or anergy of antigen-specific CD8+ T cells[48].

A number of strategies have emerged to enhance T cell responses to DNA vaccines in humans. The use of plasmids encoding both antigen and adjuvant with the aim of enhancing DC recruitment, activation and antigen cross-presentation is one such approach. For example, co-encoding GM-CSF with vaccine antigen has been shown to enhance T cell responses against HIV[49], encephalomyocarditis virus[50] and hepatitis C virus antigens[51]. Alternatively, APC transfection *in vivo* can be improved by use of nanoparticle- or liposomal-mediated delivery as discussed above[52], or by gene gun and electroporation[53]. Also, quantitative antigen expression by APC can be increased by codon-optimisation and the removal of prokaryotic sequences from DNA plasmids.

Despite these improvements, it is important to note that both T cell responses and antibody responses in humans remain inferior to those seen with recombinant viral vector or protein-adjuvant based approaches respectively. Perhaps the most promising use of DNA vaccines is in combination with viral vectors in prime-boost regimens (discussed below), which results in greater T cell and antibody responses generated by either modality in isolation.

1.1.5 Viral Vector Vaccines

Viruses have evolved efficient mechanisms for infecting cells and exploiting cellular machinery for the production of viral proteins. As such they are natural candidates for gene delivery for vaccination. Many viral species have been evaluated as recombinant vaccine vectors. These include adenoviruses, adeno-associated viruses (AAV), poxviruses, alphaviruses, measles virus, poliovirus, and HBV. The main characteristics, advantages and disadvantages of these are shown in Table 1-1.

Many of the viral vectors that have reached clinical trials were conceived for gene therapy wherein an immune response against the transgene product was undesirable. Attempts to

abrogate immunogenicity for use in gene therapy, and amplify it for use in vaccines, has led to an in depth characterization of the intrinsic vector properties that determine their immunogenicity and efficacy as vaccines. These key attributes encompass many of the immunogenic properties of the other vaccination modalities hitherto described and are discussed in more detail below.

The tropism of viral vectors for immune and non-immune cells

The ability to transduce professional APC such as dendritic cells (DC) and macrophages varies between different vectors and is determined by a number of factors including envelope glycoprotein interaction with APCs and host restriction factors that have evolved to limit viral gene expression and/or integration after membrane fusion. The former can often be readily modified by envelope pseudotyping, wherein the native viral vector envelope glycoprotein is substituted for one which ensures tropism for the desired target cell. For example, the vesicular stomatitis virus (VSV) appears to have a marked tropism for dendritic cells which can be exploited through use of VSV vectors, or pseudotyping of retroviral or lentiviral vectors (LV) with the surface glycoprotein (VSV-G). Sindbis virus glycoprotein binds to the DC-SIGN protein which confers high specificity for DC transduction *in vivo*, a property recently exploited in Sindbis glycoprotein pseudotyped lentiviral vaccines to generate HIV-1 GAG-specific immunity[54]. Accordingly, a high tropism for non-immune cells – common to many viral vectors developed for gene therapy – can be detrimental to the efficacy of viral vectors as vaccines since widespread antigen expression in the absence of co-stimulatory molecule can generate T cell tolerance. Hepatocyte-specific expression of AAV delivered transgenes, for example, appears to favour a tolerogenic immune response to encoded antigen[55,56].

Table 1-1. Viral vectors as vaccines

Virus	Genome	Main advantages	Main disadvantages	Current Clinical trials
Adenovirus	dsDNA	High titers, episomal, high transduction efficiency of APCs, safe in human trials.	Pre-exisiting anti-vector immunity (can be circumvented with chimpanzee adenovector strains)	HIV[57], Malaria[58], SARS[59], Ebola[60], influenza[61]
Poxvirus	dsDNA	Large packaging capacity, non-integrating, safe in human trials, no pre-existing immunity (fowlpox), transduce APCs.	Anti-vector immunity (e.g. Vaccinia), expensive and complex production, unstable recombinants.	Malaria[62], HIV[63]
Vesicular stomatitis virus	ssRNA-	High levels of transgene expression, low pre-existing immunity.	Safety concerns regarding neural tropism.	None
Adeno-associated virus	ssDNA	Non-pathogenic parental virus, safe in human trials.	Low packaging capacity, anti-vector immunity, (disputed) risk of integration.	HIV[64]
Alphavirus	ssRNA+	Non-integrating, high antigen expression, transduce APCs, no pre-existing immunity.	Low/moderate packaging capacity.	None
Herpes virus	dsDNA	Broad tropism and transduces APCs.	Safety concerns regarding neural tropism, pre-existing immunity.	Glioblastoma[65]
Measles virus	ssRNA-	Mucosal delivery, non-integrating.	Pre-existing immunity.	None

Poliovirus	ssRNA+	Known efficacy in children as a homologous vaccine.	Unstable recombinants, pre-existing immunity, low packaging capacity.	None
Hepatitis B virus	Partial dsDNA	High level of stable antigen expression.	Pre-existing immunity, low packaging capacity.	None
Lentiviral vectors	ssRNA+	High packaging capacity, stable antigen expression, no pre-existing immunity.	Theoretical risk of insertional mutagenesis, low inherent immunogenicity.	HIV[66]

APC activation by viral vectors

Co-stimulation and cytokine signaling between APC and T cells determine the degree of T cell activation, clonal expansion and final memory pool size and phenotype. It has long been established that for optimal T cell responses to vaccination, dendritic cells need to be activated in order to increase MHC and co-stimulatory molecule expression. TCR engagement in the absence of co-stimulation leads to anergy wherein antigen-specific T cells become unresponsive to further stimuli (non-proliferative and no IL-2 production) even with subsequent co-stimulation.

Co-stimulatory molecules are important in both early T cell activation (e.g. CD80 and CD86 with CD28, CD40 with CD40L)[67] and later differentiation into memory populations after contraction of the initial effector response (e.g. 4-1BBL and 4-1BB, OX40 and OX40L)[686970]. Studies in knockout animals have shown CD4+ T cell effector responses are more dependent upon CD80/86:CD28 co-stimulation than CD8+ T cells[71,72], whilst the TNF receptor family of ligands and receptors (4-1BB/4-1BBL, OX40L/OX40, CD40L/CD40) seem crucial for CD8+ T cell memory differentiation. 4-1BBL$^{-/-}$ mice develop grossly reduced CD8+ T cell memory pools after influenza infection but initial expansion of CD8+ and CD4+ T cells is preserved[73]. This may be because 4-1BB signaling down-regulates the pro-apoptotic molecule BIM in T cells. Similarly, in CD40L deficient mice challenged with LCMV, the expansion phase is normal but the contraction phase is prolonged and results in ten-fold fewer memory CD8+ T cells[74]. Survival signals delivered by tumour necrosis factor (TNF) receptor-ligand interactions therefore prolong the expansion phase and boost the final memory pool. This is crucial during prolonged or severe infection wherein T cells must survive long enough to clear infection[75].

In addition to T cell receptor (TCR)-MHC engagement and interaction between surface co-stimulatory molecules and their receptors, cytokine secretion by activated APCs provide a crucial third signal for T cell activation. IL-12 and type I interferon α/β (IFNα/β) are the

principal mediators of this third signal in CD8+ T cell activation and these have a critical role in preventing T cell deletion or anergy. Indeed, IL-12 alone can replace adjuvant for ensuring an immunizing rather than a tolerising response to peptide[76]. IL-12 alone cannot replace the need for adjuvant for effective CD4+ T cell clonal expansion, but recent evidence suggests IL-1 can act directly on CD4+ T cells to enhance expansion[77]. Thus effective T cell vaccines must not only generate APCs that express co-stimulatory molecules but also secretion of cytokines which ensure T cell survival and proliferation.

Viral vectors are detected by three classes of PRR in mammalian cells – TLR, retinoid acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide oligomerisation domain (NOD)-like receptors. In antigen-presenting cells, these receptors recognize a number of pathogen-associated molecular patterns (PAMPs) and initiate signaling cascades that lead to APC activation, resulting increased class I and class II antigen presentation and up-regulation of co-stimulatory molecule expression. The pattern and degree of stimulation of these PRRs determines the pattern and degree of co-stimulation and antigen presentation which ultimately shapes the T cell response. Adenovectors, for example stimulate potent type I IFN secretion in macrophages in a TLR-9- and myd88-dependent manner. DsDNA delivered by adenovectors is also capable of instigating a type I interferon response in a MyD88 independent manner and IRF-3 dependent manner (downstream of RIG-1). Some vectors, including vaccina virus (VV) and HSV vectors may block DC maturation or actively down-regulate co-stimulatory molecule expression[78–81]. This does not appear to hinder their immunogenicity, however, which may rely heavily upon cross-presentation of antigen by local non-infected DC, activated by cell debris released from necrotic cells after cytopathic infection. Loss of membrane integrity releases intracellular "damage-associated molecular patterns" (DAMPs), which include IL-1α, uric acid, DNA, mitochondrial content, and ATP, many of which directly stimulate the same PRRs that recognise PAMPs[82].

Endogenous antigen processing and multi-specific T cell responses

Antigen processing through the MHC class I pathway of dendritic cells is crucial for T cell responses. Once processed, naive T cells recognize peptides derived from foreign antigens presented on class I (to CD8+ T cells) or class II (to CD4+ T cells) histocompatibility antigens. The former bind peptides that are 8-11 amino acids in length and the latter 10-18 amino acids. These peptides bind to their respective histocompatibilty antigens through specific anchor residues, thus not every sequential peptide derived from an antigen can be presented. Estimates vary of the number of presentable epitopes within viral antigens according to a given HLA type. For example, in HIV there is approximately one CD4 epitope for every 100 amino acids and one CD8+ epitope for every 200-300 amino acids. Immune systems have evolved to process and present epitopes that are highly conserved amongst circulating pathogens with high efficiency, yet these immunodominant epitopes will vary according to allelic distribution of histocompatibility antigens. Thus T cell vaccines can elicit highly variable responses between individuals. Furthermore, pathogens with high rates of mutation can undergo T cell escape, even on several occasions within the same individual. Assarson *et al* recently characterised the T cell response of HLA-typed subjects to a large panel of candidate CD8+ T cell influenza epitopes predicted by HLA-binding and sequence conservation[83]. This showed the majority of such epitopes were from internal viral proteins and many of them were highly conserved (nucleoprotein (NP), matrix protein 1 (M1) and the polymerase basic 1 (PB1) subunit of the polymerase). Nevertheless, each of the 44 subjects recognized a unique set of peptides. In a similar study, Lee *et al* characterised H5N1 eptiope recognition across HLA types and found considerable breadth of reactivity against the length of internal virion proteins[84]. Together these studies go some way towards allaying the concern that CD8+ T cell vaccines would apply selective pressure on CTL epitopes, as long as they include multiple T cell epitopes.

T cell vaccines which express large inserts incorporating several T cell epitopes thus have the greatest chance of accommodating epitopes corresponding to multiple

histocompatibility types and generating a multi-specific T cell response less sensitive to individual epitope mutation. Live attenuated or live recombinant vaccines containing the full antigen profile of an organism ensure the greatest accommodation of HLA types and multispecific responses. At the other extreme, delivery of a single CD8+ peptide with adjuvant will leave considerable room for T cell escape.

The ideal T cell vaccine modality would thus need to ensure sustained, endogenous expression of whole or multiple antigens by APC to ensure maximal class I presentation to CD8+ T cells across a broad range of epitopes, thus limiting the potential for T cell escape. Since professional APCs are capable of cross-presentation, all vaccination modalities, including protein and adjuvant, can deliver protein to class I processing pathways in APC to some degree. Influenza (and by inference, live attenuated influenza vaccines) can enter DC and synthesise some viral proteins enabling classical loading onto class I molecules[85,86]. However, this often results in instigation of DC apoptosis so antigen presentation is short-lived. It is likely that T cell responses to influenza are principally generated by cross-presentation of antigens from cytopathically infected epithelial cells since direct DC infection is not required for T cell responses to influenza. Viral vector vaccines have a high capacity for incorporation of multiple epitopes or whole antigens and since many transduce APCs, they can ensure endogenous expression and processing of a broad range of epitopes.

However, one of the additional drawbacks of multiple epitope inclusion in an influenza vaccine is the generation of immunodominance hierarchies, wherein CD8+ T cells specific to a small number of epitopes are dramatically over-represented in the T cell response of an individual depending on their HLA type. This is particularly apparent in inbred mouse strain models after influenza infection or vaccination with whole influenza antigens[87], whilst in human influenza the most studied example is the HLA-A2 restricted $M1_{58-66}$ epitope. The underlying mechanism of immunodominance is multifactorial, involving in the first instance relative epitope abundance, preferential processing of epitopes within a given antigen, MHC-binding affinity and the number of epitope-specific naïve precursors. The subsequent expansion of very few of these epitope specificities of CD8+T cells in the

primary response is probably governed by competition for survival and proliferation signals from antigen and APC[88,89]. If a vaccine excludes an epitope from the parent pathogen that is dominant in an individual with a particular HLA type, the memory T cell response will compete with an immunodominant primary T cell response upon infection at the level of antigen presentation, with the higher affinity immunodominant epitope prevailing. Vaccines would ideally represent a range of the most dominant T cell epitopes across a range of common HLA types, but also including HLA-specific sub-dominant epitopes that limit the risk of viral escape in the event of a mutation that alters the hierarchy.

Particularly in viral vector vaccines, there is an additional risk that an immunodominant epitope in the vaccine vector itself may "drown" responses to the target antigen. In this regard, non-replicative viral vectors have a significant advantage over replication – competent viral vectors since viral proteins are only transiently cross-presented after a single round of APC transduction.

Level and persistence of transgene expression

The amount and duration of antigen exposure during T cell priming influence primary effector and T cell memory population size. T cell differentiation from naïve to effector status does not take place after a single interaction with antigen presenting cells, but during a series of encounters with multiple APCs over several days as T cells re-circulate[90]. CD4+ T cells, for example, require a minimum of 6 hours antigen exposure for activation, but in the context of minimal co-stimulation this increases to 20 hours[91]. Even after 48hrs of antigen stimulation a high proportion of naïve CD4+ cells have not yet committed to a TH-1 or TH-2 effector function[92]. Some have determined that at least 5 days of antigen stimulation are required for optimal CD4+ T cell expansion[93]. CD8+ T cells are less sensitive to duration of antigen presentation, being able to give rise to 7-10 cell divisions after 2-24 hours of antigen encounter[94–96]. Nevertheless, the peak of the CD8+ T cell effector

response is greatly enhanced by antigen persistence. This has been demonstrated by infecting mice with vaccinia strains that express varying amounts of OVA, revealing the magnitude of responding CTL population is proportional to epitope abundance[97]. Given that naïve CD8+ T cell precursor recruitment was recently shown to be highly efficient and complete even in the presence of very small amounts of antigen[98], it seems epitope abundance must have an ongoing direct effect on daughter progeny of proliferating effector T cells - either enhancing survival or proliferation - in order to boost clonal expansion and minimize contraction. It therefore remains unclear to what degree this effect is mediated indirectly through CD4+ T cell help.

The level and persistence of antigen expression by a viral vector is determined to some degree by the choice of promoters driving transgene expression but also by a number of vector-specific factors, including whether the viral vector is replicating or non-replicating, cytopathic or non-cytopathic, or delivers an integrating or non-integrating transgene. Replicating and cytopathic viral vectors, such as many VSV vectors, may induce release of heat shock proteins and other DAMPs together with high antigen loads, which ensures cross-presentation by activated DC and a robust T cell response. However, the absence of these factors may be adequately compensated for by the persistent endogenous antigen production and processing achieved by -replicating, non-cytolytic vectors. Indeed, comparison between OVA-expressing WT VSV vectors and VSV in which only cytolytic factors have been removed revealed few differences in short and long-term OVA-specific T cell immune responses[99].

Anti-vector immunity

Immune responses directed against the vector rather than encoded antigen can have a significant effect on the duration of antigen production and/or the subsequent use of the same vector as a vaccine. Similarly, prior infection with herpes, measles and adenoviruses may result in pre-existing immunity that precludes effective use of vectors derived from

these viruses. This has been extensively studied in adenovectors, the most commonly used viral vector in clinical vaccine trials. Most humans have neutralizing antibodies against AdHu5, the most widely used vaccine platform, due to exposure since early childhood and this negatively impacts their immunogencicty as vaccines[100]. Safety concerns over the use of AdHu5 in humans followed the findings of the STEP trial in which a candidate Adhu5 vector vaccine expressing HIV proteins was assessed. More individuals who had higher titres of pre-existing adenovirus antibodies became infected with HIV after vaccination than those receiving placebo. One potential explanation for this is that the pre-existing anti-adenovirus immunity was boosted by vaccination resulting in an increase in mucosal-homing memory CD4+ T cells susceptible to HIV infection[101]. Use of adenovectors isolated from chimpanzee populations (AdC) may have circumvented this problem, paving the way for their recent use in recent human malarial vaccine trials[58].

Not all viral vectors, however, appear to be susceptible to anti-vector immunity. Immune responses to modified vaccinia Ankara (MVA) virus appear to be preserved despite prior small pox vaccination[102] and VSV-G pseudotyped lentiviral vectors readily generate anti-VSV-G antibody responses but these do not appear to impede immune responses to subsequent VSV-G pseudotyped LV vaccination, although the efficacy of vaccination declines with multiple further administrations in line with increasing anti-VSV-G antibody titres[103].

Immunogenicity in prime-boost combinations

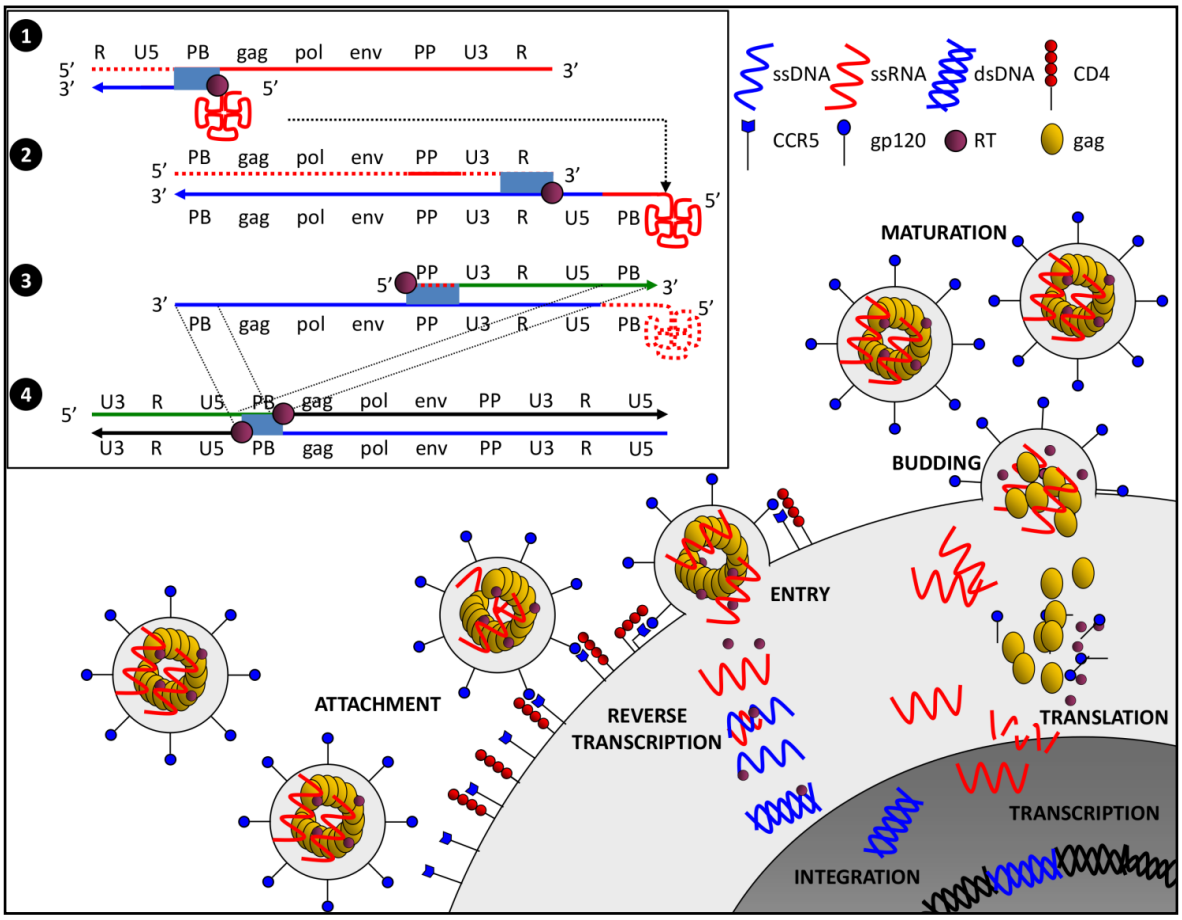
The concept of heterologous prime-boost vaccination arose with the discovery that vaccination with plasmid DNA followed by a viral vector generated more potent immune responses against a shared antigen than homologous dual vaccination or if the vaccinations were given in the reverse order[104]. Many preclinical and clinical trials of heterologous prime-boost vaccinations have since encompassed multiple vaccination modalities (bacterial, viral, DNA and recombinant protein), including the 16,000 participant STEP trial of HIV vaccination described above[105] which used a pox vector and

recombinant protein boost. The immunological basis for increased immune responses in prime boost combinations is incompletely understood. A component of this phenomenon is attributable to the fact that secondary T cell responses are typically greater and longer-lasting than primary responses due to the increased proliferative capacity of memory T cells. This does not explain, however, the additional benefit to the immune response conferred by use of different vaccination modalities for priming and recall. DNA is consistently more efficient as the prime, perhaps ensuring the immune response is focused on a single encoded antigen and not the polyclonal expansion of T cells with specificities against multiple vector components which may have to compete for survival signals and antigen exposure[106].

1.2 Lentiviral vectors for vaccination

1.2.1 Lentivirus attenuation and vector design

Lentiviruses are single-strand RNA(+) viruses of the *Retroviridae* family. This includes human, simian, equine and feline immunodeficiency viruses (HIV, SIV, EIV and FIV) but the first is the most commonly adapted for vectored delivery of genes in humans. The life cycle of HIV-1 and lentiviral vectors are compared in Figures 1-1 and 1-2. The HIV genome has been engineered to remove its replication capacity whilst preserving the ability to transfer and integrate into the host genome. This was achieved by separating trans-acting elements, encoding enzymatic, envelope, accessory and structural proteins, from cis-acting elements required for vector RNA synthesis, reverse transcription, integration and packaging.



❶ The tRNA (rose) which hybridises to the PB site provides a hydroxyl group for initiation of reverse transcription by reverse transcriptase (RT) in a 3' to 5' direction. While a ssDNA strand is produced, the RNAse H function of RT degrades the 5' end of the ssRNA. ❷ The DNA-tRNA hybrid hybridises with the R region at the 3' end continuing first strand synthesis. The PP site is resistant to RNAse activity and forms the primer to initiate the second strand synthesis. ❸ Both the PP site and the tRNA are degraded during 5' to 3' second strand synthesis. ❹ 1st and 2nd strands hybridise at their PB sites at 5' and 3' ends respectively. ❺ The DNA polymerase function of RT completes both strands. Each is now flanked by U3-R-U5 sequences, or long terminal repeats (LTRs).

Figure 1-1. HIV life cycle and stages of reverse transcription (inset). ***Ss*** single stranded, ***ds*** double stranded, ***gag*** group antigens (encodes capsid, matrix, nucleocapsid and p6 proteins), ***pol*** enzymes, ***env*** envelope proteins, ***PP*** polypurine tract, ***PB*** primer binding site, ***U3*** 3' unique sequence, ***U5*** 5' unique sequence, ***tRNA*** transfer RNA, ***RNAse H*** ribonuclease H, ***RT*** reverse transcriptase.

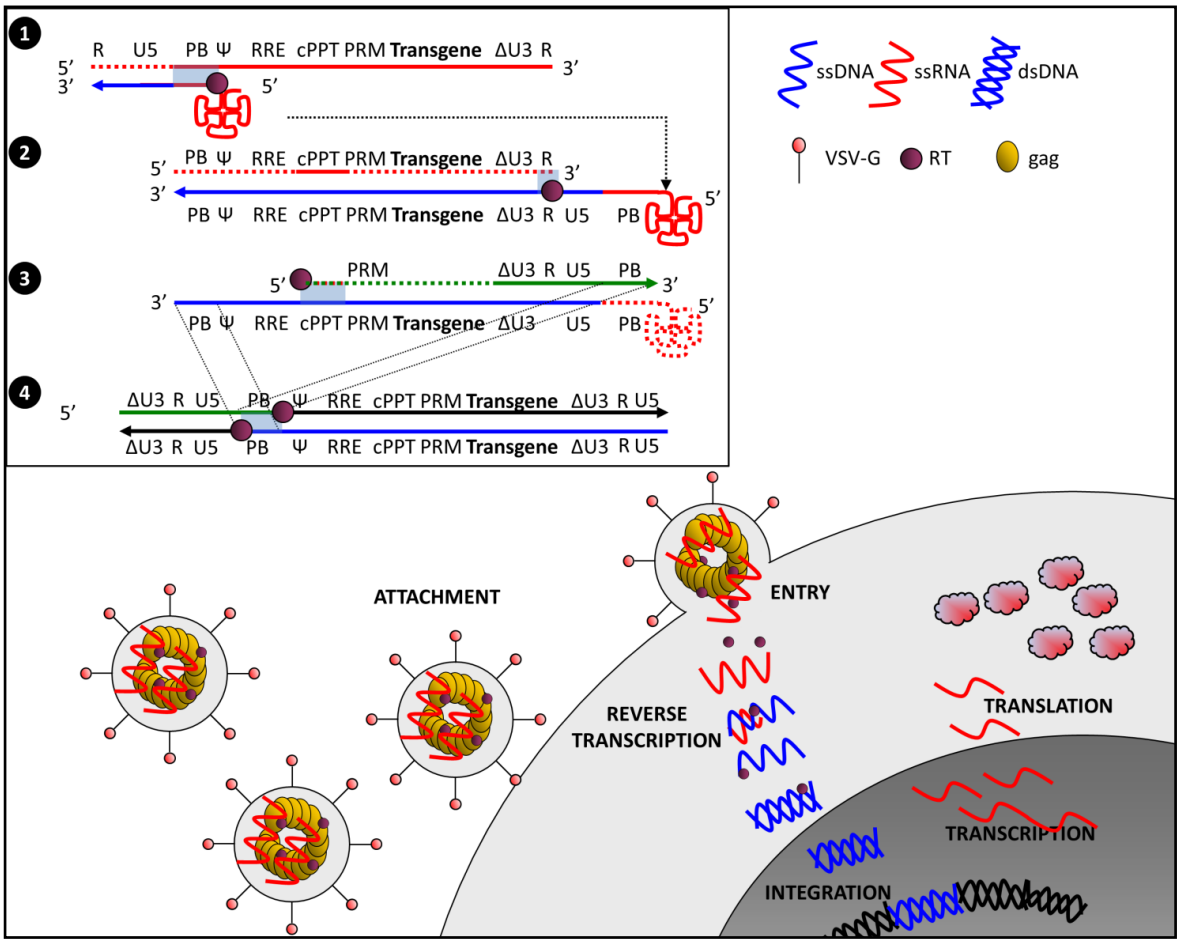


Figure 1-2. Lentiviral vector transduction. VSV-G attaches and fuses to the membrane through an unknown receptor. Because gag, pol, rev and other essential viral elements are deleted from the expression vector replications does not occur. A mutated U3 is copied across to the 5' position during reverse transcription, abrogating the promoter function of the 5' LTR and preventing transcription of the whole vector genome which would permit uptake into wild-type virions in a subject infected with HIV. Abbreviations are described in Figure 1-1.

By transiently co-transfecting producer cells with these elements on separate expression plasmids the risk of generating replication competent lentiviral vectors is greatly reduced since it would require at least three recombination events between the separated elements. Secondly, the removal of HIV pathogenicity factors (Vpr, Vif, Vpu, Nef and Tat) which are dispensable for delivery and integration of the transgene further enhanced LV safety. Thirdly, the transcriptional elements in the long terminal repeats (LTR) that flank the HIV genome have been modified to generate self-inactivating (SIN*)* vectors. This was achieved by deletion of transcriptional activation regions of the 3' U3 region of the long-terminal repeat (ΔU3). This deletion is copied to the 5' LTR of the proviral DNA during reverse transcription (Figure 1-2) where, following integration, the deletion prevents transcription of the full length vector into RNA for viral particle incorporation. This reduces the likelihood of generating a replication competent LV by recombination events, avoids transcriptional interference between the 5' LTR and internal vector promoters and also abrogates the risk of uptake of an engineered vector by wild-type HIV infection of the host cell[107]. Finally, separation of the rev element (necessary for viral packaging) onto a separate plasmid has been instituted as a further safety measure in 3rd generation LV.

LV are thus produced by transient transfection of producer 293T cells, a kidney fibroblast cell line, with up to 4 plasmids as described in Figure 1-3:

1. Transfer Vector plasmid
2. Envelope plasmid. Many glycoproteins are used to pseudotype LV. VSV-G is a commonly used option since it ensures high titres, is robust enough to withstand ultracentrifugation during concentration and has a wide target cell tropism (although the receptor for VSV-G is unknown).
3. Packaging plasmid
4. Rev plasmid (3rd generation LV only)

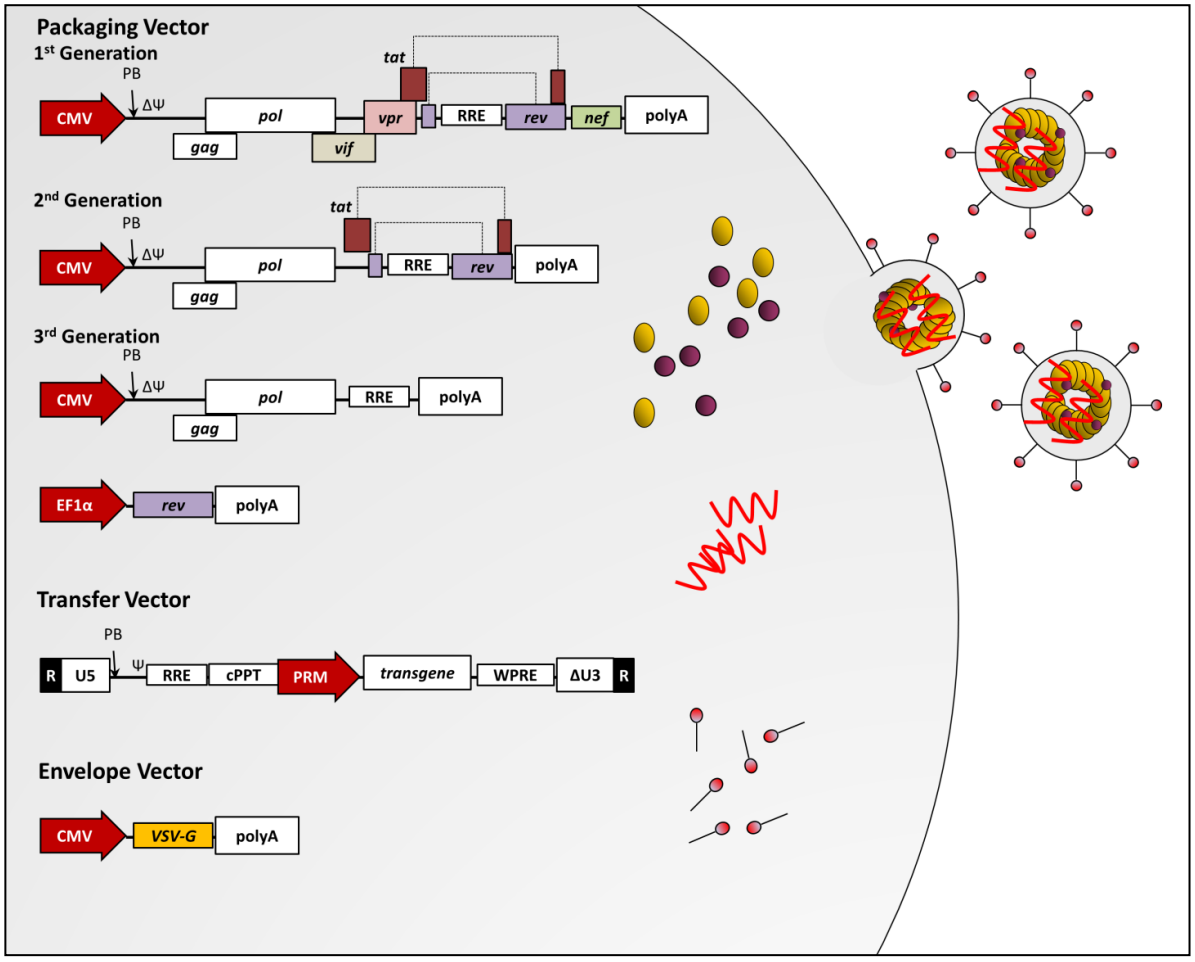


Figure 1-3 Lentiviral vector production. LV are produced by co-transfection of 293T cells with plasmids encoding packaging, transfer and envelope proteins. ***RRE*** rev response element, ***PRM*** promoter, ***WPRE*** woodchuck hepatitis virus posttranscriptional regulatory element, ***cPPT*** central polypurine tract, ***Ψ*** packaging sequence.

1.2.2 Lentiviral vectors as vaccines for cancer and infection

There are multiple examples of LV vaccines tested for tumour immunotherapy in animal models but the application of LV vaccines for prophylactic T cell mediated immunity against infection is in its infancy. Most attention in this area has focused on the potential of LV vaccine in T cell-mediated prevention against HIV, with the assumption that vector tropism and the combination of anti-vector and anti-transgene responses will together generate a memory response ideally tailored against wild-type virus.

Experiments in anti-tumour and infection vaccination using LV, summarised in Table 1-2 and Table 1-3, have consistently demonstrated humoral and T cell responses with meaningful functional correlates of therapy or protection. These studies, together with a significant recent expansion in the use of LV for gene therapy, have elucidated numerous attributes of LV that contribute to the generation of potent T cell responses by the mechanisms previously described.

1.2.3 Tropism for immune and non-immune cells

LV transduce human and mouse DC with high efficiency and this is often linked to the natural tropism of HIV for dendritic cells and macrophages. There is now extensive evidence of HIV adaptation to infection of DC as a means of onward transmission to CD4+ T cells (reviewed in[112]). However, the parent virus attributes that favour this tropism are, for the most part, removed from the attenuated vector. For example, the HIV envelope protein gp120 interacts with DC-SIGN (DC) or the mannose binding protein binding (macrophages) and is also thought to be critical for down-modulating autophagy of HIV by DC through activation of mTOR[113].

Table 1-2. Lentiviral vectors as vaccines for tumour immunotherapy in mouse models

Disease/Model	Reference	Modifications	Characterisation of immune response	Functional assays
Melanoma	[114]	None	CD8+ and CD4++ response against Melan-A	*In vivo* cytotoxicity assay
	[115]	None	CD8+ T cell response against NY-ESO-1	Elimination of *in vivo* cytotoxicity assay targets; response improved by boosting
	[116]	Hsp70 co-expressed	CD8+ T cell response against Trp2	Decreased growth of small established B16 or G-26 tumours
	[117]	None	CD8+ T cell response against Melan-A	*Ex vivo* and *in vivo* cytotoxicity assays Secondary response to challenge with peptide
	[118]	MHC II promoter for APC specificity	CD8+ T cell response against TRP-2	Increase in survival following tumour challenge with B16 cells using CMV promoter but not with MHC-II promoter
	[119]	None	CD8+ and CD4+ T cell and antibody response against NY-ESO-1	No T cell function assessment
	[120]	DC-specific promoter	CD8+ and CD4+ T cell response against NY-ESO-1	No T cell function assessment
	[121]	None	CD8+ T cell response against TRP-1	Elimination of targets in *in vivo* cytotoxicity assay Protection against B16 tumour challenge Inhibition of growth of established B16 tumours
OVA-expressing tumours	[122]	None	CD8+ and CD4+ T cell response against OVA	Elimination of targets in *in vivo* cytotoxicity assay Inhibition of growth of established EG.7 tumours

Disease/Model	Reference	Modifications	Characterisation of immune response	Functional assays
	[123]	None	CD8+ T cell response against OVA	Elimination of targets in *in vivo* cytotoxicity assay Protection against B16-OVA tumour challenge
	[124]	None	CD8+ and CD4+ T cell and antibody response against OVA	Complete protection against EG.7 tumour challenge
	[125]	vFLIP co-expressed	CD8+ and CD4+ T cell and antibody response against OVA	Complete protection against EG.7 tumour challenge
	[126]	vFLIP, MKK6 co-expressed	CD8+ T cell response against OVA	Partial regression and increased survival of mice bearing EG.7 tumours.
	[127]	MKK6, JNK1 activator co-expressed	CD8+ T cell responses against OVA	Increased survival of mice bearing EG.7 tumours.
CEA expressing tumours	[128]		CD8+ and CD4+ T cell and antibody response against CEA	Temporary regression of carcinoembryonic antigen (CEA)-expressing tumours

Table 1-3. Lentiviral vectors as vaccines for infection

Disease/Model	Reference	Modifications	Characterisation of immune response	Outcome
HPV	[129]	E7 fused to calreticulin to enhance MHC I presentation	CD8+ T cell and antibody response against E7	No functional T cell assessment
West Nile Virus	[130]	None	Antibody response againt WNV E-glycoprotein	Protective, sterilizing humoral immunity against WNV
Influenza	[131]	Non-integrating LV	CD8+ T cell response against Matrix-1 protein	No functional T cell assessment or infectious challenge
HSV-1 and HSV -2	[132]	(FIV vector)	CD8+ T cell response and Antibody response against HSV glycoprotein B	Increased survival to both HSV-1 and HSV-2 challenge
HIV	[133]	Multiple HIV epitopes encoded	Multispecific CD8+ and CD4+ response against multiple encoded HIV epitopes	*In vivo* cytotoxicity assay
	[134]	Codon-optimisied gp120. Integrase deficient vector	CD8++ T cell response and sntibody response against gp120	*In vivo* cytotoxicity assay
	[135]	Codon-optimised gp120	CD4+ and CD8+ T cell response	No functional T cell assessment
	[110]	Prime-boost with Adhu5 vector	CD4+ and CD8+ + T cell response against Gag, Pol and Rev	No functional T cell assessment
SIV	[136]	Prime-boost with LV with non-cross reactive VSV-G pseutoypes	CD8++ and CD4++ T cell response against SIV GAG	Partial protection against intra-rectal SIV challenge in primates

Despite the removal of these parent virus adaptations to DC infection, transduction of human and mouse DC by VSV-G pseudotyped LV is retained with efficiencies ranging from 35-90% [137–140,123,141,142]. Importantly, LV-transduced DC remain viable, present antigen, respond to maturation stimuli and are capable of secreting cytokines such as IL-12[143]. In direct comparison with adenovectors, LV have been shown to be 2-10 times more effective in transducing mouse and human DC[144]. Importantly, DC transduction has been demonstrated *in vivo* in murine models and is integral to CD8+ T cell responses. Skin-derived DC are transduced by subcutaneous vaccination of mice with LV and subsequently readily identified in draining lymph nodes. Adoptive transfer of these *in vivo* transduced DC from lymph nodes into recipient mice elicit potent CD8+ T cell responses to encoded antigen[145]. Intravenous injection of LV also transduces high numbers of proliferation-capable DC precursors and macrophages[146].

Although human DC transduction by VSV-G pseudotyped LV has been demonstrated *in vitro*, it occurs at efficiencies much lower than that observed in mice. This may be in part due to reduced DC tropism of VSV-G compared with native HIV envelope gp120 and can be addressed by use of alternative pseudotyping with envelopes which interact with DC-SIGN such as Sindbis virus envelope[54] or the haemagluttinin and fusion proteins of measles virus[147]. However, LV transduction of human DC is further restricted by sterile alpha motif domain and HD domain containing protein (SAMHD1) which inhibits the replication of LV in human dendritic cells by hydrolyzing the cellular deoxynucleotide triphosphates (dNTPs) below the level required for reverse transcription[148]. Lentiviral vectors that incorporate the simian immunodeficiency virus (SIV) accessory protein Vpx, which degrades SAMHD1 by initiating its ubiquitination, have very recently been shown to transduce human DC with much higher efficiency than current HIV-1 derived vectors[149].

As with other viral vectors, the relative efficiency of transduction of APC and non-APCs may influence the resultant T cell response by encouraging stimulatory and regulatory responses respectively. Like AAV, LV transduce hepatocytes with high efficiency which are capable of inducing T cell tolerance to presented antigen. LV transgene expression can be de-targeted from DC by use of a hepatocyte-specific promoter and addition of 3' microRNA targets which limit expression in haematopoetic cell lineages. This

induces prolonged expression of encoded antigen together with transgene product-specific T cell deletion or exhaustion[150]. Whilst this is an advantage for gene therapy, it is undesirable in vaccination which may conversely benefit from better means of targeting APC and de-targeting non-immune cells. Pseudotyping LV with a single chain antibody targeting MHC-II has recently achieved this, demonstrating highly specific transduction of MHC-II expressing cells after intravenous LV administration[151].

1.2.4 The innate immune response to lentiviral vector components

One of the reasons LV have come to the fore in gene therapy in recent years is their low immunogenicity, avoiding strong PRR stimulation and thus minimizing anti-transgene CD8+ T cell immune responses. This would appear to make LV a poor choice as a T cell vaccine since the presentation of antigen in the absence of co-stimulation by APCs risks T cell deletion or anergy rather than activation. However, LV do instigate at least transient DC activation by stimulation of TLR7 and then TLR3 stimulation as delivered ssRNA is reverse transcribed[152], although this may occur predominantly at high MOI *in vitro*[153]. This, together with efficient transduction and endogenous antigen production and processing seems to consistently ensure strong CD8+ T cell responses to LV encoding antigen alone.

The ability of LV to incorporate large or multiple inserts means that additional immunostimulatory signals such as DC maturation signals or specific co-stimulatory molecules can be readily delivered to APCs in order to enhance T cell activation. Examples of such signals used to date in LV vaccines include CD40L[154], heat-shock protein 70 (hsp70)[155] and myD88 or TRIF-1[156]. This group has previously demonstrated that transduction of DC with LV expressing activators of signaling pathways involved in maturation can dramatically enhance co-stimulatory molecule expression and cytokine secretion thereby enhancing T cell responses against co-encoded antigen. One particularly promising approach has been NFκB pathway activation by viral flice-like inhibitor protein (vFLIP) from Kaposi's sarcoma herpes virus (KHSV)[157]. Transduction of bone-marrow derived dendritic cells from mice with LV expressing vFLIP led to marked up-regulation of CD80, CD86, CD40 and intercellular adhesion molecule 1 (ICAM-1) and

increased TNFα and IL-12 secretion to levels comparable to lipopolsaccharide (LPS) stimulation. When vFLIP was co-expressed with OVA, antigen specific CD8+T cell responses were enhanced 10-fold and vFLIP-OVA vaccinated mice demonstrated improved tumour-free survival in an OVA-expressing tumour model. Stimulation of the p38 mitogen-activated protein kinase (MAPK) pathway by an LV encoded MAPK Kinase 6 (MKK6) mutant that constitutively phosphorylates p38 also results in DC co-stimulatory molecule up-regulation (although no increase in pro-inflammatory cytokines) and enhanced T cell responses against co-encoded antigen[158].

An alternative strategy to enhance DC activation by LV vaccines is to down regulate pathways that inhibit DC activation. LV encoding shRNA against A20, an inhibitor of NFκB activation, can enhance NFκB signaling and DC activation, resulting in A20 resulting in co-stimulatory molecule and pro-inflammatory responses[159]. Specific inhibition of co-inhibitory signals may also enhance T cell responses to LV-transduced DC. Transduction of DC with LV co-encoding shRNA against PDL-1 and OVA resulted in generation of OVA-specific hyper-activated CD8+ T cells without antigen-mediated T cell receptor down regulation[160].

1.2.5 Level and persistence of antigen expression

Since the LV genome is integrated into the target genome it is both more stable than episomal DNA and also propagated with subsequent cell divisions, ensuring high-level, persistent antigen expression. Other modifications have been made to LV since their inception to further improve transgene expression. For example, the addition of a cPPT enhances transport of the vector into the host cell nucleus after transduction[161]. Inclusion of the WPRE increases transgene expression (as it does in many vectors by an unknown mechanism). This ensures both high quantity and duration of antigen expression which, as discussed above, is critical for optimizing T cell memory and protection. Our group has recently examined the ramifications of long-term antigen expression induced by intravenous administration of LV. This route of administration transduces high numbers of proliferation-capable DC precursors which ensures antigen presentation for up to 2 months. Long-term expression of OVA was slightly lower than

green fluorescent protein (GFP) which may reflect the higher immunogenicity of this antigen. Nevertheless, OT-1 CD8+ T cells (specific for an OVA epitope) underwent proliferation when adoptively transferred up to 3 months after recipient mouse vaccination, and *in vivo* cytotoxic killing of OVA peptide-pulsed splenocytes was preserved at this time point, allaying the concern that persistent antigen expression by LV may induce tolerance[162]. It is unclear whether or not the prolonged antigen presentation following LV vaccination sustains persistence of T cell memory, although there is some evidence that T cell memory populations are longer-lasting than those induced by vaccinia vectors in which antigen presentation is transient[123].

1.2.6 Anti-vector immunity

Immune responses directed against LV envelope glycoproteins can inhibit effective re-use of the vector. However, although neutralising antibodies against VSV-G have been demonstrated in mice following VSV-G pseudotyped LV vaccination, these have not been shown to block transduction after subsequent LV administration. Repeated subcutaneous vaccination with VSV-G pseudotyped LV in mice induces minimal anti-vector neutralising antibody[163] and *in vitro*, DC can be readily transduced additively with consistent efficiency[164]. There is some evidence that VSV-G activates human complement which can then inhibit vector fusion with target cells, a problem that can be circumvented by PEGylation of VSV-G pseudotyped LV[165].

1.2.7 Prime-boost vaccination regimens

The inclusion of LV in vaccination regimens with other modalities is a relatively un-tested area. A second homologous vaccination of macaques primed with an HIV-1 derived LV expressing SIV GAG induced secondary T cell responses of greater magnitude and longer persistence than seen in the primary response after single vaccination, and this corresponded to greater protection against SIV challenge. Notably, this study used LV pseudotyped with different VSV-G serotypes to avoid the theoretical risk of anti-vector immunity described above[166]. Others have demonstrated that heterologous vaccine regimens incorporating an LV expressing GAG, Pol and Rev

(VRX1023) and Adhu5 encoding GAG and Pol (VRC5409) significantly improved the percentage of polyfunctional HIV-specific CD4+ and CD8+ T cells compared with dual vaccination of either vaccine, with Adhu5 prime-LV boost being the most effective strategy[167].

1.3 Comparing vaccine modalities for T cell responses against infection

Quantifying the T cell response to vaccination typically involves one or more of the following surrogate measures:

a) **Enzyme-linked immunospot assay (ELIspot)** of number of IFNγ-secreting splenocytes (in mouse experiments) or peripheral blood mononuclear cells (PBMCs, in human experiments) following peptide re-stimulation *in vitro*.
b) I**ntracelluar cytokine staining of T cells** from splenocytes/PBMC for IFNγ, IL-2 and TNF as corollaries of functionality after stimulation *in vitro* with relevant peptide.
c) **Tetramer or pentamer staining** for numbers of antigen-specific CD8+(and increasingly CD4) T cells.
d) **^{3}H-thymidine** and Carboxyfluorescein Succinimidyl Ester (CFSE) proliferation assays
e) **Killing/cytotoxicity assays**

Despite routine measurement of T cell number or function, there are no defined thresholds that need to be achieved for vaccine efficacy, nor are these readily predicted. These parameters are also subject to non-standardised experimental variations which confound comparison between studies. Live infectious challenge, for example, would seem an appropriate benchmark for vaccine efficacy. However, influenza challenge of mice, for example, only results in a predictable clinical sequelae (such as weight loss or survival) if virus strain and challenge dose are standardised. In the absence of an agreed standard challenge strain or dose there is considerable variation in the "robustness" of challenge. Inoculation dose and replication efficiency (V(0) and π_α respectively in Figure 1-5, page 70) affect peak viral titres in different

ways. Increasing the inoculation dose will not increase peak viral titres but will shift it to an earlier time point, whereas increasing replication efficiency (by using a mouse-adapted strain versus a non-adapted strain, for example) will enhance peak viral titres but will not change its timing[168]. The measured impact of a memory T cell response following vaccination will be highly sensitive to both these variations. Other factors that contribute to variation between studies include the species of mouse used (BALB/c and C57BL/6 mice, for example, have varying susceptibilities to identical influenza strains) and the route of administration (intratracheal administration delivers a greater inoculation dose to the alveoli than intranasal).

Perhaps the greatest variable that confounds comparisons of immunogenicity between candidate T cell vaccines is that the T cell response is dynamic, and there is no agreed time-point after vaccination at which to apply the above measures of efficacy. There are three distinct phases to primary T cell responses: expansion, contraction and memory. Expansion follows interaction between the T cell receptor (TCR) on naïve T cells and its cognate MHC-peptide complex on the surface of professional antigen-presenting cells with APCs in lymphoid tissue, providing there are sufficient co-stimulatory and cytokine signals to meet the threshold for T cell activation. Following an expansion phase and antigen clearance, a contraction phase begins driven by rapid effector T cell apoptosis. This population does not dwindle completely and plateaus at a level (usually several-fold lower than the peak of expansion) that can be maintained for considerable lengths of time. These memory T cells have altered trafficking properties, are less susceptible to apoptosis and can respond rapidly to future antigen encounter without the need for co-stimulatory signals. Conventionally, responses to T cell vaccines are usually measured after the expansion and contraction phases (during which T cell number changes rapidly at different time points) and during the memory phase, but there is significant debate as to when this begins. Indeed it is impossible to determine without dynamic monitoring of a response to a specific vaccine. The delay between vaccination and immunological assessment (or infectious challenge) is usually a compromise between the point at which the contraction phase has finished and the practical constraint of completing series of experiments within a limited period of available time.

These variables thus confound the comparison of different modalities of vaccination in terms of their efficacy in generating T cell responses. This problem is compounded by a paucity of studies comparing two or more vaccination modalities in the same experimental system. Some 30 years into T cell vaccine development it is therefore impossible to state with confidence which of the modalities for vaccination discussed above (or in which combination) generate the greatest degree of T cell mediated protection in any given disease model.

A more fruitful approach may be to consider the requirements of an ideal T cell vaccine for controlling a given disease and tailor a chosen vaccine modality accordingly. Lentiviral vectors have some specific advantages in this regard. Their low inherent immunogencicity means that the immunological benefit of encoded adjuvants over and above the immunogenicity of the vector is more easily determined. Their, their high capacity for transgene incorporation means combinations of immunomodulating factors can be included and their ability to transduce non-dividing cells means a functionally wide range of cells can be harnessed for a desired immunological outcome.

This thesis describes the testing of a lentiviral vector vaccine in a mouse model of influenza. This was chosen, in part, because influenza has a fast-changing antigenic profile that rapidly eludes antibody mediated protection and as such is a prime candidate for T cell vaccine strategies that target conserved epitopes. However, influenza is an acute, potentially lethal viral infection, which replicates rapidly and at a site distant from secondary lymphoid organs in which memory T cells reside. Furthermore, T cell responses in the lung are tightly regulated to protect the delicate architecture from the deleterious consequences of inflammation. These disease-specific factors present robust challenges to a T cell vaccine and are considered in more detail in the next section.

1.4 Influenza virology and immunology

In broad terms, those who search for a universal vaccine against influenza that can protect against multiple strains can be divided into two camps. Some groups have attempted to generate broadly cross-neutralizing antibodies against conserved B-cell epitopes on the surface of the influenza virion. Others have tried to generate potent T cell responses against influenza epitopes, principally towards internal components which are highly conserved.

Both these approaches to universal vaccination have a long history, benefiting from and developing in parallel with significant advances in the understanding of influenza structure and life-cycle, in-host viral dynamics, the immune response to influenza (and other respiratory infections) and the development of effective vaccine modalities for generating more potent antibody and T cell responses.

1.4.1 Influenza structure, life cycle and in-host viral dynamics

A number of features of the influenza structure and life cycle are relevant to effective vaccine design. In principle, proteins that are exposed at the surface of the virion are amenable to antibody attachment for sterile immunity whereas those which are internal can only be targeted through T cell recognition of processed epitopes. This is an oversimplification, however, since generating antibody responses to internal components of the influenza virion has been shown to confer partial protection, by mechanisms that remain unclear[169]. An important distinction between external and internal proteins of the influenza virus is that many of the former are subject to wide-ranging and dynamic sequence and structural variations between and within virus subtypes, whereas the latter tend to be more conserved due to functional constraints upon their essential role in viral assembly and therefore viability. This informs the selection of conserved T cell and antibody epitopes which might be targeted for cross-strain protection through vaccination.

An understanding of the influenza life-cycle (**Error! Not a valid bookmark self-reference.**) is integral to accurate modelling of the dynamics of influenza infection and the immune response. This has significant repercussions for vaccination measures designed to modify rather than prevent infection.

Viral Structure and host cell entry

Influenza A is an enveloped orthomyxovirus with a single-stranded, negative-sense RNA composed of 8 segments. These encode 11 viral components, namely HA, NA, NP, non-structural protein (NSP1), non-structural protein 2(NSP2), M1, Matrix 2(M2), polymerase acidic protein (PA) and polymerase basic proteins 1, 2 and F1 (PB1, PB2, F1). HA consists of two domains, HA1 and HA2, and associates into trimers which form spikes on the virion surface. The interaction of HA1 with sialic acid on the cells surface is specific to the type of linkage formed between sialic acids and the glycoproteins on the membrane surface[170]. The two predominant linkages are α(2,3) and α(2,6). Viruses capable of infecting humans recognise the α(2,6) linkage, whereas avian and equine influenza strains bind to the α(2,3) linkage. Swine express both linkage types on the epithelial surface making them an ideal mixing vessel for avian and human influenza strains. Interspecies variation in the phenotype and distribution of sialic acid receptors throughout the respiratory tract means both the route of administration (e.g. intratracheal versus intranasal) and strain of an influenza virus will influence its behaviour in a given species – a major consideration in the extrapolation of findings from animal influenza challenges to humans.

Binding of HA2 to the sialic acid linkage triggers uptake into endosomes wherein the low pH exposes the HA2 fusion domain which inserts into the endosomal membrane and precipitates virus/endosomal membrane fusion. This process leads to a conformational change in the M2 ion channel, allowing acidification of the viral core and releasing viral ribonucleoproteins (NP, PA, PB1, and PB2) into the host cell cytoplasm[171]. This critical function of the M2 ion channel means it is structurally conserved between viral strains. Together with its partially external location, this makes it an attractive target for antibody based approaches to universal vaccination.

Viral replication and transcription

Viral replication and transcription occur in the nucleus since they are dependent on many host factors abundant in nuclei. The viral ribonucleoproteins (vRNP) thus have nuclear localisation signals that ensure their trafficking into the nucleus via cellular nuclear import machinery[172].

Viral RNA dependent RNA polymerase (RdRp, a combination of PB1, PB2 and PA) initiates replication by RdRp after "self-priming" of the negative sense (-) RNA genome by hybridisation of 5' and 3' ends, which have partial inverse complementarity[173]. A key feature of viral replication is that is it highly error-prone due to the absence of an RNA proof-reading enzyme, leading to an error rate of approximately 1 in 10,000 nucleotides. Given that this is the approximate length of the influenza RNA, most replication by RdRp will generate a mutant. This results in the rapid antigenic drift that causes much of the seasonal variation in influenza and presents a problem for vaccination that targets variable domains.

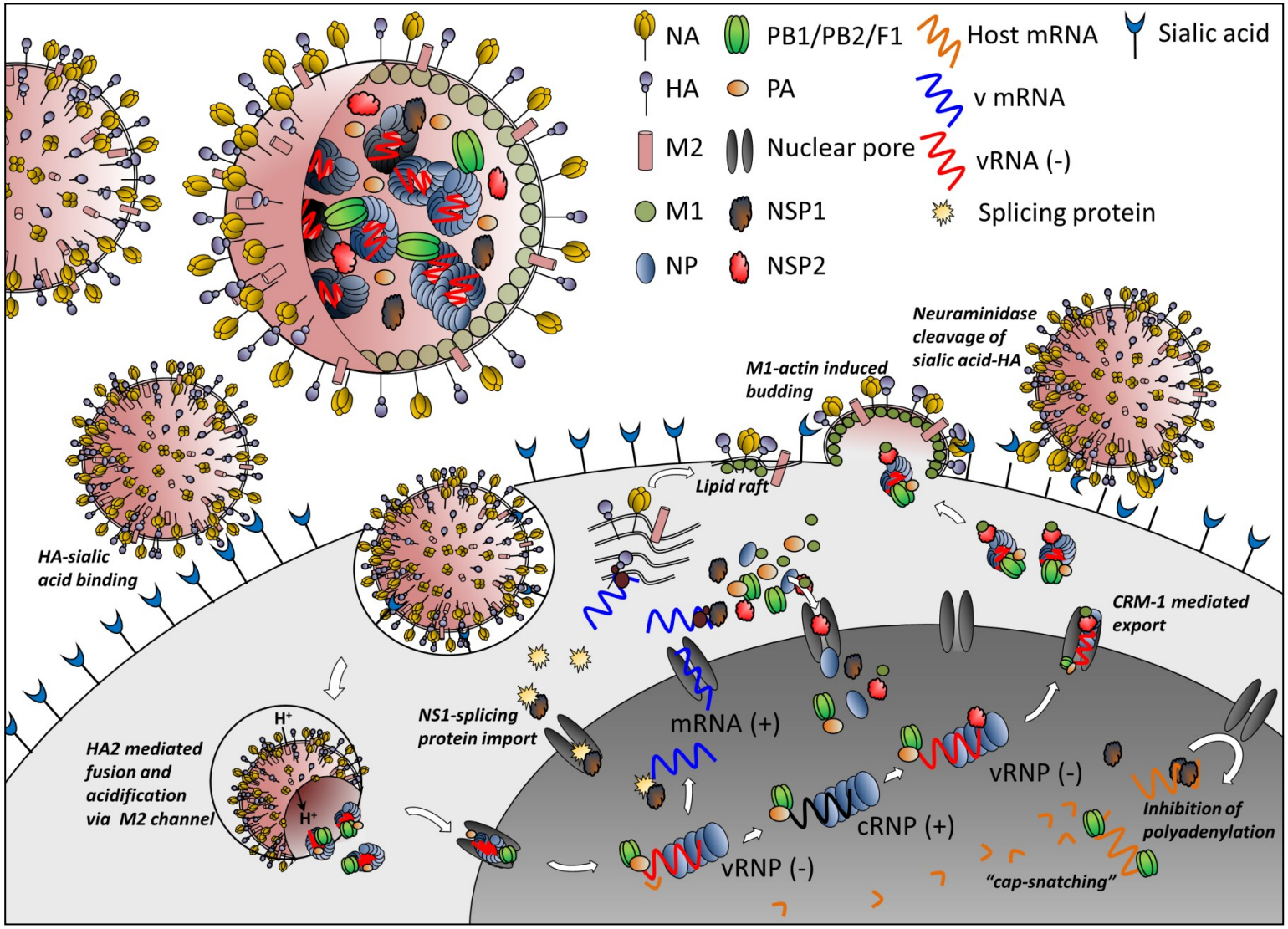


Figure 1-4. The influenza life cycle.

NA neurominidase

HA haemagluttinin

M2 Matrix 2 (channel)

M1 Matrix 1

NP nucleoprotein

PB polymerase basic protein

PA polymerase acidic

NSP non-structural protein

RNP ribonucleoprotein

Transcription of viral mRNA from the (-) RNA is primed by 5' methylated "caps" cleaved from cellular mRNAs by the endonuclease activity of PB2, a mechanism known as "cap-snatching"[174] which substantially inhibits host cell mRNA translation. Viral mRNA transcripts from segment 7 and 8 undergo splicing to encode 2 protein products each (M1, M2 and NSP1, NSP2 respectively) and this exploits cellular splicing machinery, again at the expense of host mRNA splicing[175]. NS1 binds to splicing proteins via associated U6 small nuclear RNAs causing them to relocate to the host cell nucleus depriving cytoplasm cellular mRNAs of splicing[176]. Through binding to both the cleavage and polyadenylation specificity factor (CPSF) and polyA binding protein II (PABPII), NS1 prevents the cleavage of cellular mRNA at the polyadenylation site in preparation for addition of the polyA polymerase[177]. NS1 inhibits nuclear export of cellular mRNAs through preventing their proper polyadenylation. Polyadenylation of viral mRNA, however, requires neither a classic polyadenylation signal nor PolyA polymerasse but it achieved through reiterative copying of 5-7 uracil residues close to the 5' end by RdRp which "stutters" back and forth over this U-rich region[178].

In summary, in addition to inhibiting cellular mRNA splicing, the influenza virus inhibits 5' capping and polyadenylation of host mRNA, thus reducing its stability, nuclear export, excision of the 5' proximal intron and translation initiation. Viral protein synthesis is promoted at the expense of the host cell, permitting fast, high quantity replication and producing approximately 200 virions per infected cell per day[179]. Whilst hijacking host cell translational machinery will also reduce the innate immune response to infection (such as type I interferon production) it also has profound cytopathic effects resulting in cell death, independently of a cytotoxic T cell response. Indeed, apoptosis rates are directly proportional to viral mRNA synthesis[180]. Depletion of susceptible target cells can be a major limiting factor in sustaining viral replication therefore influenza strains must evolve an optimal compromise between replicative vigour and prolongation of infection to ensure onward spread through viral shedding. This has implications for the "systems biology" modelling of influenza spread and estimating the population impact of measures such as anti-viral medications and T cell mediated protection, both of which limit the duration and severity of disease rather than confer sterile immunity.

Nuclear Export

vRNPs associated with negative sense RNA are exported through nuclear pores by the chromosome region maintenance 1 protein (CRM-1) pathway. It is thought that NSP-2 (otherwise known as nuclear export protein) interacts with CRM-1 and also the the N-terminus of M1. In turn, M1 interacts with the RNA(-) associated ribonucleoproteins through its C-terminus. Through this "daisy-chain" of viral proteins the ribonucleoproteins are exported from the nucleus. Since the binding of NSP-2 to M1 masks the latter's nuclear import signal this ensures unidirectional transport of the vRNP-RNA complex into the cytoplasm for assembly and budding[181].

Viral Assembly and Budding

Virions bud from the apical surface of infected cells after accumulation of the envelope proteins (HA, NA and M2) at this site and these are therefore the only viral proteins which have a surface-exposed component. HA, NA and M2 associate with lipid rafts in the membrane lipid bilayer and the cytoplasmic tail of M2 is thought to be critical in the association of other internal proteins at this site[182]. It remains uncertain whether genomic RNA is packaged stochastically into virions during assembly[183] or if this occurs in a deterministic fashion[184], but the identification of numerous packaging signals in a number of coding and non-coding regions of influenza RNA segments supports the latter hypothesis[185]. The segmental nature of the influenza RNA genome means that cells infected with virus of more than one strain may readily reassort to create daughter progeny with unique antigen profiles and pathogenicity characteristics. In broad terms, this risks a combination of HA and/or NA epitopes from a non-human strain - for which there is little or no cognate humoral immunity in the human population - with segments encoding internal viral components which are structurally well-adapted for human infection. Such antigen shifts have been responsible for at least two pandemics in the past century (H2N2 in 1957 and H3N2 in 1968). Population prevalence studies of influenza genotypes suggest at least 2-3 such reassortment viruses emerge per annum, with a preference for single segment reassortments of NA or HA[186].

The M1 protein is crucial for viral budding and cooperates with several cellular factors to achieve this, including host cortical actin microfilaments[187]. The final stage of viral release is mediated by NA which cleaves the sialic acid residues (bound to membrane glyocoproteins and glycolipids) which attach to HA preventing viral separation from the host membrane[188]. Sialic acid binding to HA is crucial to viral entry and its cleavage during viral budding by NA may play a role in limiting viral superinfection of host cells.

1.4.2 The dynamics of influenza propagation and the primary immune response

The primary immune response to influenza

The innate immune response to influenza infection is initiated by the release of pro-inflammatory cytokines by antigen presenting cells and infected epithelial cells. Infected macrophages secrete macrophage inflammatory protein 1α (MIP-1α), MIP-1β, RANTES (regulated on activation, normal T cell expressed and secreted), monocyte chemoattractant protein-1 (MCP-1), MCP-3, MIP-3α and IP-10 whilst epithelial cells secrete RANTES, MACP-1 and IL-8[189]. These appear to have a crucial role in the recruitment of mononuclear cells of the adaptive immune response in both mice and humans and are considered in more detail in Section 4.3.4. Immediate control of viral replication, however, is provided by the production of type I interferons (IFNα/β) by both infected epithelial cells and macrophages[190–192]. This slows viral replication and renders epithelial cells more resistant to infection, delaying productive infection and allowing time for the adaptive immune system to respond. Mice bearing a disrupted IFNα/β receptor demonstrate accelerated viral growth kinetics after challenge with a non-lethal A/X31 (H3N2) strain and develop much higher compensatory peak antibody titres[193].

Both B cell and T cell adaptive immune responses are thought to determine the end of viral expansion and eventual clearance of influenza virus. Virus-specific antibodies appear at around day 5 after infection in mice with a peak in IgM titres between day 8-10 and IgG at day 25[194]. Early antibody isotype depletion studies in mice established

that IgG and IgA, but not IgM are dispensable to antibody-mediated viral clearance during the primary response[191]. This is thought to occur by both reducing the attachment of virus to host cells (through anti-HA antibodies) and preventing budding virus from detaching from host cells (anti-NA antibodies)[195].

The primary CD8+ and CD4+ T cell response is also not detectable in the lung until around day 5 of infection. Suppression of the CD4+ T cell response in mouse models of influenza (through antibody-mediated CD4+ T cell depletion or use of MHC class II knock-out mice) only slightly delays clearance and has little impact on survival[196,197]. Removal of the CD8+ T cell response, on the other hand, significantly delays clearance and increases mortality, although some mice are able to clear infection if CD4+ T cell and B cells are intact[198]. If both CD4+ and CD8+ T cell responses are depleted, however, animals generally do not control infection despite substantial production of anti-influenza IgM driven by higher viral loads[199]. Similarly, animals depleted of both B cells and CD8+ T cells typically cannot control infection and do not survive[200–202]. Taken together, these studies suggest crucial roles for CD8+ T cell responses and IgM antibody responses to clear primary infection, but a lesser role for CD4+ T cell responses.

Modelling influenza in-host dynamics

Studies of lymphocyte depleted mice or knockout models have several limitations, not least that near-complete removal of one arm of the adaptive immune system will have unpredictable, non-physiological consequences for the remaining components. Furthermore, these studies provide qualitative rather than quantitative insights into the impact of the immune response. In recent years there has been a proliferation of experiments that combine high frequency quantitative sampling of virus load and immune parameters together with mathematical modelling in order to gain insights in to the complex interaction and relative impact of the different components of the immune response upon influenza in-host dynamics. This has been facilitated by increasing computer processing power but also by experience and knowledge gained from modelling the in-host dynamics of other viral infections such as HIV. This

approach has also benefited from growing interest in anti-viral measures that do not rely upon antibody mediated sterile immunity, such as the increasing use of anti-viral drugs and emergent clinical trials of T cell based vaccines and from growing understanding of influenza virus pathogenicity factors. Justification of such approaches relies upon their impact on disease duration and severity in the individual, which in turn will impact on infectivity and spread throughout a population.

To date, such models have generated disparate conclusions. For example, one analysis of viral load data from infected human volunteers has shown that it is possible to describe the infection dynamics without inclusion of the immune response as a modifying factor[203,204]. This study suggests the interesting conclusion that infection might be limited by exhaustion of available primary epithelial target cells rather than immunomodulation. However, this would require complete desquamation of the upper respiratory tract (URT) which, although observed in ferrets exposed to an epidemic strain in a 1930s study[205], is not observed in humans with seasonal flu. It also fails to explain the well-established phenomenon that immunocompromised patients shed influenza for prolonged periods[206207,]. One problem with modelling the human response to influenza is that it is limited to use of mild strains, analysis of peripheral blood rather than lung immune responses and the use of non-invasive measures of viral load (such as in expectorated mucus) which are subject to greater variation than whole-lung measurement in the mouse. Mice do not provide a model of infection relevant to URT infection in humans, partly because it is difficult to specifically administer virus to the URT without distal aspiration and partly because sialic acid residues that interact with human strain are mostly expressed in distal respiratory epithelium.

The ferret provides a more accurate model, since the distribution and subtype of sialic acid receptors for human and avian influenza viruses is very similar to that found in humans[199] and restricted inoculation of the URT is technically straightforward. This model has recently been used to compare the spatiotemporal dynamics of three current influenza strains of varying pathogenicity and anatomical tropism in some detail: avian H5N1, seasonal H3N2 and pandemic H1N1. In ferrets, seasonal H3N2 is quickly cleared from the URT after peaking at day 1 post-infection, whereas avian H5N1 infects the alveoli and peaks at day 3[208]. However, quantification of the immune

response in this study was crude, limited by the relative scarcity of antibodies and reagents for analysis of cellular immune responses in the ferret compared with the mouse.

Mice do provide a relevant model of flu strains that infect the lower respiratory tract in humans (such as H5N1 avian influenza or severe pandemic 1918 Spanish influenza). To date, much of the modelling analysis of influenza propagation and its modulation by immune responses is based upon data from two studies of experimental infection of BALB/c mice from over 20[209] and 30[210] years ago. The former study data are of viral loads in wild-type and nude mice (which lack T cells and have a severely impaired B-cell response). The latter dataset reports viral load, type 1 IFN levels and antibody titres and tracks infection both in immunocompetent mice and IgM-, IgG- and IgA-depleted mice. One recent analysis of these data found that both the type I IFN response (as a surrogate of the innate immune response) and antibody response need to be taken into account to adequately explain observed patterns of viral propagation and clearance[211].

A more complete attempt to model virus propagation and the adaptive immune response was described recently, using higher resolution sampling (up to 12-hourly) of mice infected with H3N2 (A/X31) influenza, with detailed analyses of the contemporaneous CD4+ T cell, CD8+ T cell, and isotype-specific antibody responses[212]. Viral titres peaked between days 2 and 3, with complete clearance by day 11. As in previous studies, virus-specific CD8+ T cells were not detectable before day 5 and peaked between days 9 and 11. Virus-specific IgM levels also peaked at this time-point, returning to baseline after 20 days, whilst IgG levels peaked at day 25, decaying slowly over the next 60 days.

A series of differential equations incorporating these measured parameters together with a number of calculated parameters (such as number of uninfected epithelial cells at day 0) and fixed assumed constants (for example, rate of virus production per infected cell) were combined with estimated parameters (e.g. the rate at which CD8+ T cells kill infected cells or IgM antibody neutralizes free virus) and then tested to generate a model that best fits the observed data. The interrelationship between these measured, calculated, assumed and estimated parameters is shown in Figure 1-5.

The model of best fit reveals a number of interesting insights:

- Peak viral load is target cell limited. There is an early and rapid drop in the number of cells susceptible to infection which cannot be accounted for by exhaustion of available cells, or by the adaptive immune response. This is most likely due to the effects of IFNα/β on increasing epithelial cell resilience to infection. This early pre-adaptive phase of virus control corresponds to plateauing of viral loads at around day 2 to 3 (when adaptive responses are negligible).

- Peak viral titres occur earlier but are not increased if viral infectivity or inoculum dose is enhanced. Increasing replication rate, however, dramatically increases peak viral load.

- Viral titres start to drop with the arrival of CD8+ T cells and the appearance of IgM antibody in the adaptive phase. By removing CD8+ T cells and/or antibody responses from the model and determining how this impairs the fit (submodel analysis), the degree to which these contribute to viral clearance can be estimated. In the best fit model, these appear to make equivalent contributions to viral clearance.

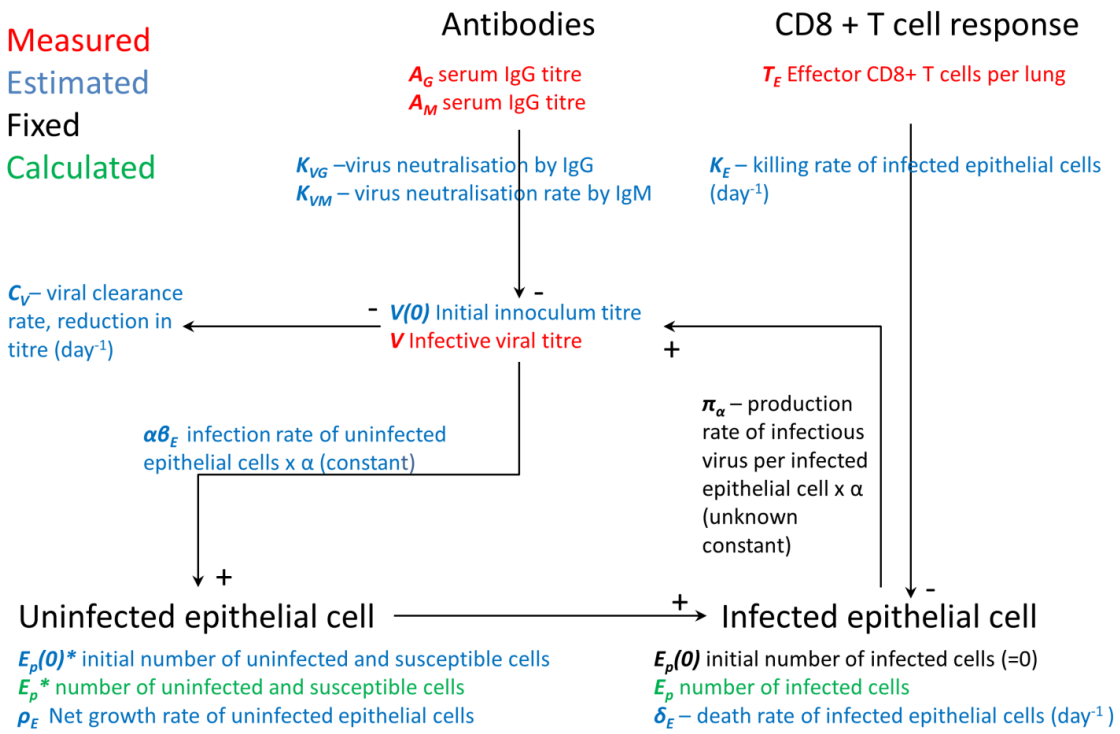


Figure 1-5 Modelling in-host influenza dynamics.

Modelling the impact of T cell vaccines against influenza

Similar models have been used to attempt to describe the impact of pre-existing T cells in the lung at the point of challenge upon viral dynamics, as might occur after previous exposure to a heterosubtypic influenza strain (as discussed below). As one may assume, these models predict more rapid viral clearance and lower peak titres if CD8+ T cells are present in the lung at the onset of infection. Surprisingly, however, any starting number of CD8+ T cells less than 10^5 seems to be insufficient to have any impact on the peak viral load or clearance time[213]. The killing rate of infected cells has to be very high to impact upon the rapidly rising viral loads seen in the first few days of infection, when the type I IFN response has yet to impact upon target cell susceptibility to infection. This is a crucial, if often overlooked, factor in estimating the likely impact of generating systemic T cell immunity against influenza as a vaccination strategy. Whilst the presence of systemic T cell memory, for example, may bring forward the time at which peak CD8+ T cell numbers are achieved in the lung by 2-3 days, this may have negligible impact on peak viral titres and therefore the disease severity of a highly pathogenic strain.

However, since these models are based on quantitative measurements of the *primary* T cell response they do not account for the improved ability of memory influenza-specific T cells in the secondary response to expand and kill upon reencounter with antigen[214], and should therefore be interpreted with caution. More importantly, such models focus on viral load and clearance without addressing key parameters of clinical severity or duration of disease which can also be readily quantified (weight loss, respiratory rate, mortality). This is crucial since the clinical severity of influenza infection is a complex, composite function of not only viral titres and their cytopathic effect but also immune-mediated injury. Establishing the relative contribution of these two factors to lung injury in humans is further confounded by their interdependence. The CD8+ T cell response, for example, is driven by antigen load, yet this direction of influence is notably absent from current schemata of virus-immune system interaction models (Figure 1-5). T cells also have a direct role in tissue injury independently of viral load. The clearest demonstration of this is the observation of deleterious effects on

respiratory parenchyma in the absence of replicating virus when inducible, alveolar-restricted expression of an antigen is followed by transfer of antigen-specific T cells[215].

Therefore, in order to determine the clinical impact of vaccines that generate T cell responses against influenza, we require more quantitative data regarding the dynamics of the *secondary* T cell response and more qualitative information about how virus and immune response interact to generate clinicopathological sequelae.

1.4.3 Heterosubtypic immunity and the T cell response to influenza

T cells and heterosubtypic immunity

It has been known since the late 1970s that T cells are capable of conferring cross-strain protection against influenza. Mice immunised intraperitoneally with a single strain of influenza A would generate potent populations of splenic T cells capable of killing target cells *in vitro* that were infected with other influenza A strains[216,217]. This was the first convincing mechanism to explain the phenomenon of heterosubtypic immunity against influenza A that had been observed more than 10 years earlier[218], wherein mice exposed to one influenza A strain were found to be partially immune to exposure to a another. A number of subsequent studies demonstrated that intravenous adoptive transfer of NP-specific CD8+ T cells could confer heterosubtypic immunity to mice[219–221] and further evidence followed of a reduction in heterosubtypic immunity following CD8+ (and to a lesser extent CD4+) T cell depletion[222].

It has proved harder to demonstrate a role for T cells in retrospective studies of protection from influenza epidemics in humans due to unknown prior exposure history and variation in non-adaptive susceptibility factors. In 1983 McMichael *et al* demonstrated through experimental infection of human subjects that viral shedding was reduced in the presence of cytotoxic T cell memory from prior infection, despite the absence of specific antibodies[223]. A number of subsequent epidemiological studies in the 1980s showed cross protection conferred by one influenza subtype against

another during sequential or overlapping epidemics[224] or periods of mixed outbreaks[225]. Memory T cell responses against epitopes from M1, NP and PB2 in humans following exposure to seasonal influenza strains were also identified later in this decade[226]. Since then, over 5000 experimentally-determined strain- and HLA-specific T cell epitopes have identified and are now available in publically accessible databases[227].

Indirect evidence of the importance of CD8+ T cell mediated cross-protective immunity in humans emerged more recently with the observation that mutation in the immunodominant T cell epitopes of influenza NP (normally highly conserved) can confer a selective advantage. Voeten *et al* describe the emergence of a mutation (R384G) that disrupts MHC I presentation of the HLA-B27 restricted influenza A $NP_{383-391}$ epitope in a 1993 H3N2 strain and quickly superseded strains lacking the mutation which disappeared in subsequent epidemics[228]. This pattern of emerging T cell escape influenza variants has since been demonstrated in HLA-B*3501 and HLA-B*08 restricted CTL epitopes[229,230]. Following the recent SOH1N1 pandemic, it was proposed that the mild phenotype may have been in part due to cross-protective CD8+ T cell responses generated by seasonal influenza exposure against the highly conserved HLA-A2 restricted $M1_{58-66}$ epitope shared by SOH1N1[231]. Natural immunity from seasonal influenza infection, but not seasonal influenza vaccination which generates poor T cell responses, may also generate cross-protective CD8+ T cell responses against H5N1 avian influenza A[232]. Importantly, these studies consistently demonstrate that heterosubtypic immunity from natural infection is not only partial, but starts to wane after an as yet ill-defined time point ranging from 3-9 months[233]. In mice this phenomenon has been defined more precisely with the demonstration that cross-protection from primary influenza infection starts to wane after 15 weeks[234235].

Although CD4+ T cells have an essential role in establishing long-lasting protective antibody responses[236], the depletion of naïve CD4+ T cells in mice has little or no impact on the primary response to influenza. Furthermore, memory CD4+ T cells after primary infection wane considerably faster than CD8+ T cells and before heterosubtypic immunity declines[237] and early mouse depletion studies of CD4+ T cells after primary infection appears to have significantly less impact on heterosubtypic

protection than CD8+ memory depletion[234]. Historically, therefore, there has been less interest their role in heterosubtypic immunity compared with CD8+ T cells. However, more recent studies have pointed towards a significant role for CD4+ T cell memory in heterosubtypic protection of mice and humans. Using TcR Tg mice in which CD4+ T cells recognise class II restricted HA epitopes, McKinstry *et al* generated memory CD4+ T cell population by influenza infection and then transferred these to unprimed mice[238]. This successfully protected mice from lethal influenza challenge, (although others have contended that the transfer of lower, "physiological" numbers of CD4+ T cells results in minimal protection[239]). Protection in recipients of TcR Tg CD4+ memory T cells is impaired in the absence of functional B cells and CD8+ T cells, suggesting memory CD4+ T cells confer heterosubtypic immunity through supporting the primary antibody and CD8+ T cell response. However, CD4+ T cells do suppress initial viral titres when transferred into severe combined immunodeficiency (SCID) mice (lacking B and T cells) before infectious challenge by an IFNγ-dependent mechanism, suggesting they are capable of contributing to viral control directly. Titres were only temporarily suppressed, however, and death was delayed rather than prevented. In this study, a perforin-dependent mechanism of selection of CD4+ epitope-specific viral escape mutants was also observed, raising the possibility of cytotoxic CD4+ T cells playing a role in viral control. This finding appears to corroborate a previous study by the same group showing early, antibody-independent control of viral titres by adoptively transferred CD4+ memory T cells in a perforin-dependent manner[240]. Such CD4+ secondary effector functions are only seen in lung populations (splenic CD4+ memory T cells remain predominantly cytokine-secreting and non-cytotoxic, for example) which presents a problem for human studies which are mostly limited to analysis of peripheral blood T cell populations. Nevertheless, Wilkinson *et al* recently demonstrated that in healthy volunteers challenged with mild H3N2 or H1N1 strains, symptoms were less severe and viral shedding lower in the presence of pre-existing CD4+ T cells specific to pools of NP and M1 peptides found in the challenge strains. Furthermore, circulating CD4+ T memory T cells isolated during infection showed evidence of cytotoxicity against peptide pulsed target cells in a chromium release assay.

Antibody responses and heterosubtypic immunity

Antibody responses may also have a role in heterosubtypic immunity. Dominant antibody responses emerge to the highly immunogenic haemagluttinin (HA) and neurominadase (NA) surface glycoproteins which protect completely against subsequent infection with the same strain. However, the tolerance for high mutation rates in this region without loss of function (attachment and fusion) mean there is typically little cross-neutralisation between anti-HA and anti-NA across subtypes or between drift variants within subtypes. Such subtypes are phylogenetically categorised based on their amino acid sequence. HA, for example, has 16 subtypes belonging to two broadly similar structural conformations. Despite this structural variation, there has been at least one report of anti-HA mediated cross protection in humans[241] and it has been shown that vaccination of pregnant mice resulted in cross-strain protection of their offspring against 3 different subtypes[242], dependent upon serum virus-specific neutralizing antibodies in both mother and neonates. There have since been rare reports of antibody clones isolated from humans capable of cross-neutralising two different HA subtypes. The first of these in 1993 identified an antibody clone (designated C179) that cross-reacted with both HA1 and HA2. Subsequent sequence analysis of these subtypes identified the stem region of the HA molecule as the likely binding site[243]. In 2008 an antibody designated CR6261 was isolated from a healthy, vaccinated volunteer which showed cross-neutralising activity against multiple HA types including several HA5 types (from avian H5N1) to which the individual had not previously been exposed[244]. Importantly, these antibodies were neutralizing both *in vitro* and *in vivo,* rescuing mice when given up to 5 days after lethal challenge with either H1N1 or H5N1 influenza. This pivotal nature of this study became evident with the subsequent elucidation of the epitope target of CR6261 - a portion of the HA stem highly conserved within the two major groups of the 16 HA subtypes.

Several studies have since identified similar monoclonal antibodies in individuals that recognize this region, including the important observation that broadly cross-neutralising (BCN) anti-HA stem antibodies dominated the B-cell response in nine individuals infected with the 2009 pandemic SOH1N1[245246]. One study has isolated a

monoclonal antibody by high-throughput screening of human plasma cells (using a novel single-cell culture method) from individuals recently vaccinated with the seasonal influenza vaccine which cross-neutralises group 1 and 2 HA subtypes and confers protection against influenza in mice and ferrets by passive transfer[247]. It has also been shown that BCN antibodies specific to the HA stem region are readily generated in mice and non-human primates, particularly by DNA/viral vector prime-boost combinations[248]. The recent multiple successes in identifying anti-HA stalk antibody clones from phage libraries may be explained by the increased sensitivity of detecting these by novel high-throughput techniques and use of trimeric recombinant HA for capture[249].

However, despite demonstration of neutralization activity *in vitro* and *in vivo*, BCN antibodies consistently fail to confer sterile immunity against influenza, even at high doses. In all the above-mentioned studies using vaccination to generate BCN neutralising anti-HA stalk Ab or using passive transfer of cross-reactive anti-HA stem mAb, mice suffer weight loss after inoculation (albeit less severe than controls). This is at odds with a mechanism of action that blocks viral fusion with target cells and confers sterile immunity. It is conceivable that the inhibition of the HA hinge mechanism in the HA stem region which prevents fusion is less efficient than antibody-mediated blockade of the receptor-binding region of the HA head. Indeed, neutralization assays against pseudtoyped virons reveal the doses of mAb required to achieve 100% neutralization are several orders of magnitude greater than seen in a typical neutralization assay employing mAb directed at the HA receptor binding region. This lower neutralisation efficiency may be compounded by the fact that titres of these antibodies are typically low, either in response to infection or vaccination. Taken together, these may explain why the apparent ease with which BCN antibodies are generated by seasonal vaccination or infection does not translate into subsequent protection.

Finding a means to enhance the titres of these antibodies to protective levels through vaccination is currently the focus of much effort. The low titres seen after infection or seasonal influenza vaccination may be a consequence of the immunodominant B-cell response to the HA receptor binding head which is more exposed than the HA stalk region[249]. Attempts to generate an immunodominant B-cell response against the HA

stalk through vaccination with a recombinant truncated “headless” HA have generated cross-neutralizing antibodies but have not conclusively shown these bind to the HA stem region. Vaccination did confer improved survival in mice but, as in previous experiments, did not confer sterile immunity[250]. DNA/adenovector prime boost combinations appear to enhance titres of cross-neutralizing anti-HA stem antibodies, but again these fall short of conferring sterile immunity against heterosubtypic challenge.

The current efforts to develop anti-HA stem BCN for universal protection may benefit from insights gained from previous attempts to develop antibody responses against conserved B-cell epitopes on the virion surface. Much attention has previously focused on the M2 glycoprotein as a potential trigger for antibody-mediated heterosubtypic immunity since it has an ectodomain exposed on the surface of the influenza virion (M2e) and is highly conserved. However, generation of anti-M2e antibodies by vaccination has been impeded by poor B cell responses to M2e peptide (unless fused to a more immunogenic antigen) and antibody titres in humans and animals are very low after influenza A infection[251252]. Treanor *et al* was the first to show that passive transfer of an anti-M2e monoclonal antibody accelerated influenza clearance following sublethal challenge[253]. There have been subsequent successful attempts to enhance anti-M2e antibody generation in mice with consistent demonstration of enhanced protection against lethal influenza challenge[254–258]. In human trials, Sanofi Pasteur Biologics Co reported after a Phase I trial that a recombinant vaccine consisting of M2e fused with hepatitis B core (HBc) – designated ACAM-FLU-A – was safe, well-tolerated and generated high titres of anti-M2e antibodies. This formulation was also shown to confer 70% protection in ferrets against lethal H5N1 (Vietnam 2004). However, the results have yet to be formally published some 4 years after these announcements.

There are significant parallels with the recent investigation of anti-HA stem BCN antibodies. Anti-M2e antibodies were been reported to demonstrate neutralizing activity *in vitro*, for example slowing the infection rate of cultured Madin-Derby canine kidney (MDCK) cells[259], and yet in all *in vivo* experiments immunity was non-sterilising, with shortened duration and severity of disease but no prevention. As with the target of anti-HA stem BCN, this may be because the M2e ectodomain is less accessible to

antibody shielded by the more extensive ectodomains of HA and NA, making virus neutralization inefficient.

Recent experiments have shed light on the protective mechanism of action of anti-M2e antibodies. M2e is expressed abundantly on the surface of infected cells in a manner accessible to antibody, and it has been proposed that this may mediate cytotoxic killing by natural killer (NK) cells engaging with the Fc domain of bound antibody[260], although others have found that depletion of NK cells has a limited impact on the protection conferred by anti-M2e antibody[261]. Complement mediated killing of infected cells opsonised by anti-M2e antibody also appears to be minimal. However, the depletion of alveolar macrophages appears to completely abrogate the protection conferred by anti-M2e antibodies against influenza infection, suggesting that enhanced phagocytosis of virions (or infected cells) by innate immune cells may play a crucial role in limiting the course of infection[262]. This may occur by a number of mechanisms, including reducing the pool of infectious virus, enhancing innate Type I IFN responses and increasing T cell responses through enhanced cross presentation of phagocytosed antigen. The dependence of the protectiveness of anti-M2e antibodies upon innate cellular responses has been further confirmed by the observation that anti-M2e antibodies confer no benefit in Fc receptor knock-out mice[262].

Similar findings have been observed with the protective efficacy of antibodies against influenza NP, which is highly conserved (>90%) between all influenza A strains[263]. Mechanistically, antibody response against an internal virion target cannot be neutralising, and yet passive transfer of anti-NP antibodies has been consistently shown to mediate infection-permissive protection against influenza A[264–266267,]. Like anti-M2e mediated protection, this protection appears to be dependent upon Fc receptor expression by innate immune cells, as demonstrated by its attenuation in FcR deficient mice. Furthermore, the absence of CD8+ T cells removes any survival benefit conferred by anti-NP antibodies, supporting the notion that the non-neutralising antibodies enhance viral clearance by boosting innate and cellular adaptive repsonses[268].

It is noteworthy that the role of FcR or T cell dependent mechanisms have yet to be investigated in the context of BCN antibodies directed against the HA stem, yet the

pattern of infection-permissive protection strongly suggests they may enhance immunity by a similar mechanism to anti M2e or anti-NP antibodies.

The T cell response to influenza and lung injury

T cell effector mechanisms appear to have different roles in viral clearance and lung injury raising the tantalizing prospect that specific manipulation of the immune response might enhance the former and minimise the latter. CD8+ T cells clear virally-infected cells by Fas ligand (FasL) or perforin-dependent mechanisms yet there is little evidence that this cytotoxic effector function drives parenchymal damage. This is also the case in experimental RSV infection wherein FasL and perforin seem dispensable for histopathological change[269]. CD8+ T cell derived inteferon γ (IFNγ) and tumour necrosis factor α (TNFα) appear to play a prominent role in lung injury but the clearance of influenza is only marginally impaired in their absence[11,12]. TNFα is a key mediator of lung injury in influenza infection and one study has shown CD8+ T cell derived TNFα to be the principal driver of inflammation in a model of inducible alveolar antigen expression. In this model, immunopathology is initiated by TNFα-dependent stimulation of chemokine secretion by epithelial cells and subsequent inflammatory cell recruitment, rather than by TNFα-induced cell death. IFNγ has a less clear role in lung injury, with some studies reporting that milder injury occurs with transfer of IFNγ deficient CD8+ T cells to a host expressing transgenic antigen in distal airways. Others, however, have reported enhanced injury in live influenza infection in the presence of IFNγ-deficient T cells. Taken together, these studies suggest whilst IFNγ is capable of inducing inflammatory damage, an effective IFNγ response ultimately mitigates associated inflammation and immunopathology by the rapid control of viral replication and inhibition of alternative immune cell recruitment such as eosinophils[270].

The degree to which different T cell subset responses can negatively influence the clinical outcome of influenza is highly relevant to vaccine design. This is underlined by the RSV vaccine trails in the 1960s mentioned above, in which increased morbidity and

mortality was observed in vaccinated subjects following infection. Vaccination had primed excessive CD4+ TH2 responses and eosinophilic influx on secondary challenge[7]. This was later attributed to a portion of the RSV attachment G-protein that induced potent CD4+TH2 responses; following its removal, vaccination could generate T cell mediated protection without eosinophilia. Deleterious consequences of CD4+ TH-2 responses in lung injury have been consistently demonstrated in mouse models. Intravenous adoptive transfer of influenza-specific CD4+ TH-1 T cells promotes viral clearance upon influenza challenge of mice, whilst TH-2 transfer enhanced lung pathology without accelerating viral clearance[271]. Similarly, IL-4- and IL-10-secreting CD4+ T cell responses potentiate lung injury and airway hypersensitivity in influenza infection of mice without enhancing viral clearance or protection[272]. In humans, high numbers of circulating virus-specific CD4+ T specific to NP and M1 during early influenza infection are associated with subsequent severity. Also, strong and early CD4+ TH-17 responses have been associated with a more severe illness in hospitalized patients during the recent SO H1N1 pandemic[105]. This has also been demonstrated in knockout mouse models, wherein IL-17 has been shown to be essential for lung injury, weight loss and neutrophil infiltration but dispensable for viral clearance[273,274].

1.4.4 Memory T cell responses to influenza

Memory T cells are typically divided into central and effector subsets based on their homing properties[275–277]. Central memory T cells express CD62L and CCR7 which directs their recirculation through lymphoid organs and do not have instant effector function on encounter with antigen[278] but rather expand rapidly and differentiate to supply the effector T cell population. These effector T cells express low CD62L and CCR7 and are found in non-lymphoid tissues where they respond rapidly to antigen encounter with cytotoxicity or cytokine secretion.

Not long after these distinct subsets were characterised, there were attempts to determine their relative contribution to secondary responses in the lung. It has been known since the 1970s that during the secondary response to a heterologous strain, memory cytotoxic T cells appear in appreciable numbers in the lung much earlier after

exposure than in naïve mice[279].The availability of tetramers for labelling of antigen-specific T cells in the late 1990s permitted more precise tracking of influenza-specific CD8+memory T cells and their subsets during primary and secondary influenza infection[280]. This led to two unexpected findings. The first was that the anatomical head-start afforded by cognate T cell immunity was considerable, with large numbers of antigen-specific CD8+T cells appearing in the lung around 3 days sooner in mice previously exposed to a heterologous subtype versus naïve counterparts. The second finding challenged the classical paradigm of central memory T cells residing in secondary lymphoid organs (spleen and draining lymph nodes) until being recalled and expanded at the site of infection where they differentiate into effector T cells. Instead, very large numbers of CD8+ memory T cells were found to reside in the lung parenchyma. Their distribution within the lung was not only within bronchus associated lymphoid tissue (BALT) or nasal-associated lymphoid tissue (NALT) but also in inducible regions of lymphoid aggregates arising in the perivascular or interstitial areas[281]. Some estimate as many as 40000 antigen specific T cells can be recovered 1 month after influenza infection of in mice within the airways themselves, an area previously regarded as hostile to T cells due to the presence of surfactant and proximity of the external environment[282,283]. Importantly, CD8+T cells in both the airways and parenchyma have an effector phenotype and are able to respond more rapidly to secondary infection than their splenic or lymphoid tissue counterparts, consistent with their higher expression of activation markers ($CD62L^{LO}$, $CD25^{+}$, $CD69^{+}$). Those T cells with the highest activated proportion (70-80%) were retrieved from the airways by broncho-alveolar lavage (BAL), with a progressive decline in this proportion observed through lung parenchymal T cells (50%), mediastinal lymph nodes (20%), and spleen (15%)[284]. Comparatively little is known about antigen-specific CD4+memory T cells in the airways although these have also been reported to persist in the lung airways after infection[285] albeit at much lower frequencies than CD8+ T cells.

These observations have coincided with the revelation that the differences in expandability and function between central and effector memory were not as distinct as first described[237,286]. Non-proliferating effector T cells in the lung airways may have a key role in early responses to heterologous influenza challenge[287] since they are able to respond to very low viral loads with rapid cytokine secretion but, curiously, minimal

cytolytic potential[288]. The latter may be attributable to lower expression of leukocyte function-associated antigen-1 (LFA-1), an integrin key to T cell-target cell adhesion. Interestingly, the proliferative capacity of airway memory T cells is recovered by their extraction in BAL suggesting alveolar macrophages or surfactant may actively suppress this function. Importantly, effector memory cells in the lung parenchyma have been shown to retain their ability to proliferate in response to influenza infection[289290]. These cells have high lymphocyte function-associated antigen 1 (LFA-1) expression, cytokine secretion and cytolytic activity. Central memory phenotype CD8+ T cells also reside within the lung parenchyma either in loose lymphoid aggregates or in more organized BALT and nasal associated lymphoid tissue (NALT) regions. These structures lack afferent lymphatics and instead are in direct contact with the lung epithelium allowing them to respond rapidly to infection.

Following the discovery of large, lung-resident populations of T cells after influenza infection it was shown that both spleen or mediastinal lymph nodes can be removed without detriment to the size, speed or efficacy of the response to subsequent heterosubtypic challenge[291–293]. Also, if T cell immunity was generated by intraperitoneal or intravenous inoculation of influenza, significant lung-based memory T cell populations failed to develop and heterosubtypic protection was much lower than that seen with intrapulmonary infection[294]. Furthermore, adoptive transfer of memory T cells by intranasal routes establishes more effective heterosubtypic influenza protection than intravenous transfer[288]. These experiments suggested mixed splenic or lymph node central/effector populations are, at least in the short term, redundant for effective T cell secondary responses within the lung whilst airway and lung-parenchymal memory T cells act as the principal agents of heterosubtypic immunity by controlling viral replication soon after inoculation.

During the secondary response, lung- and airway-based T cells are subsequently supported by an further influx of effector memory T cells recruited from the circulation, peaking in number around day 6[295]. These cells are non-proliferating and their recruitment is not an antigen-specific phenomenon, since intranasal administration of TLR agonists will also induce their ingress[296]. As a consequence, the fraction of antigen-specific effector memory T cells in this wave of recruitment is very

similar to the proportion of antigen-specific T cells residing in the secondary lymphoid organ and circulation. Since the pool of available effector memory T cells in secondary lymphoid organs declines with time after the primary infection, the maximum achievable peak of this accelerated influx may reduce with time and provide another explanation for why heterosubtypic immunity wanes. A third (and final) phase of the recall response involves arrival of T-memory cells that have been proliferating in response to antigen presentation in the local lymph nodes and spleen. These begin to arrive in the airways at day 4 and peak around day 7 (and can be distinguished from previous, non-replicating waves by their incorporation of intravenously administered BrdU). This wave is analogous to the primary naïve response but occurs more rapidly since T-memory cells in the lung parenchyma can proliferate more rapidly in response to antigen presentation by epithelial and non-APCs and without the requirement for co-stimulation.

Lung-based T cell persistence

The persistence of compartmentalised populations of T cells with different capabilities after primary influenza infection raises the question of how these populations are maintained. Within secondary lymphoid organs it is well established that memory T cell populations are kept at steady-state by homeostatic proliferation under the control of IL-7 and IL-15, which replaces cells at a similar rate to which they are lost[297–299]. However, the inability of airway CD8+ T cell memory populations to proliferate suggests their numbers must be maintained by recruitment from lung parenchyma or circulating populations. This is supported by the observation that LFA-1 expression is uniformLy down-regulated within 48hrs of adoptive transfer of T cells to the airways and yet after primary infection in mice a steady proportion (10-40%) are found to be LFA-1 positive up to a year later, implying recent migration into the airway compartment. Experiments with Sendai virus in mice have shown that intra-tracheal biotinylated populations of airway T cells are progressively diluted by non-biotinylated cells with time in the absence of proliferation. Also, intravenously transferred labelled memory T cells will appear in the airways within 14 days in normal mice without an

infectious stimulus[300].

Pro-inflammatory signals from the innate immune system, epithelial and endothelial cells after infection in the lung may also play a role in sustaining airway and parenchymal T cell recruitment after infection is cleared. CD11c-expressing cells are maintained in the lung and display enhanced antigen presentation for several months after the resolution of influenza or RSV infection[301,302]. This may be the consequence of IFNγ secretion by γδ T cells recruited late in infection resolution[303]. Alveolar macrophages, which have a prominent immunoregulatory role in the steady-state[304], are sustained in an activated state following infection by GM-CSF and thought to be secreted by both mesenchymal cells and memory T cells after infection[305]. This cytokine also recruits a new population of $CD11b^{HI}$ macrophages which may persist for many weeks within the airways[306]. Epithelial cells also demonstrate prolonged alterations after influenza infection, most notably up-regulated TLR3 expression and thus a lower threshold for secretion of IL-8, IL-6 and expression of ICAM-1[307]. There are thus multiple mechanisms of "innate imprinting" following infection that may last for the lifetime of these cells once activated. This not only enhances the readiness of the host for future heterologous infection but may also sustain non-specific recruitment of effector T cells long after infection has resolved[308].

The limitations of heterosubtypic immunity

Were heterosubtypic immunity to provide complete and lasting cross protection, then influenza would quickly cease to circulate in the human and animal population. Instead, the numbers of airway and lung-based T cell memory cells starts to wane soon after influenza infection, whereas splenic memory T cell populations are preserved at high levels indefinitely[280]. In humans the half-life of anti-influenza CTL activity in peripheral blood is thought to be approximately 12 months[309] and is undetectable three years after infection. Numbers start to fall at around 15 weeks after infection in mice. Airway T cells are not highly apoptotic and do not appear to re-enter the circulation. Therefore, assuming the rate of their mucociliary clearance remain constant, the decline in number can be attributed to reduced recruitment of effector T

cells from the lung parenchyma or circulation. Indeed, their decline in number is coincident with a dramatic shift from effector to central memory phenotype in antigen-specific T cells in secondary lymphoid organs[310] which supports the notion that lung-based effector memory populations are sustained over the long-term by supply from secondary lymphoid organs. It also correlates with the slow decline in innate immune and epithelial cell activation in the lung after infection[308].

The lung is a fragile tissue adapted for gas exchange which perhaps cannot sustain the prolonged presence of mutually sustaining T cell and innate immune cell populations deleterious to lung architecture and function. Bronchiectasis and sarcoidosis are testament to the potential pathological consequences of chronic and sustained parenchymal T cell responses. The declining lung CD8+ T cell memory pool after infection may therefore be a an evolved compromise between ameliorating future heterosubtypic infection and the risks of long-term airway destruction that accompany sustained T cell infiltration.

1.5 Formulating the ideal T cell vaccine against influenza

Taking into account the mechanisms of T cell mediated heterosubtypic immunity and lung injury, the ideal characteristics of a T cell vaccine against influenza can be formulated as follows:

1. **T cell phenotype:** Generates cytotoxic CD8+ T cells, TH-1 CD4+ T cells and avoids TH-2 CD4+ T cell responses to maximise viral clearance and minimise lung injury.
2. **T cell specificity:** Generates multispecific T cells against conserved virion epitopes to provide cross-strain protection and minimise the risk of T cell escape.
3. **Anatomy**: Generates a lung-based T cell memory population for early control of viral replication.

4. **Longevity:** Generates lasting central memory populations to supply sustained lung-resident effector memory T cell populations that are maintained for at least the duration of an influenza season.
5. **Clinically applicable:** Generates protection rapidly with a simple, acceptable and safe vaccination regimen.

No T cell vaccine for influenza has so far achieved the above criteria, although there has been substantial progress in each category. These advances are discussed in more detail in the introductions to the experiments described below, after a description of the molecular and immunological methods used.

2 Methods

2.1 Molecular biology techniques

2.1.1 Buffers and media

These are described in Table 2-2.

2.1.2 Polymerase chain reaction (PCR)

PCR reaction mixtures used the GoTaq® Green Master Mix (Promega, Madison, WI) and other reagents shown below in Table 2-1.

Table 2-1 PCR reaction mixtures (top) and cycle parameters (bottom).

Reactive	Stock concentration	Volume
GoTaq® Green Master Mix	2X	25
Forward Primer	10 µM	2.5
Reverse Primer	10 µM	2.5
Water	-	15
DNA Template	20 ng/µL	5
TOTAL		50

25-30 cycles were run in a Hybraid thermal cycler, using the parameters shown below

Phase	Time	Temperature
Activation	10 minutes	95º C
Denaturation	45 seconds	94º C
Primer annealing	30 seconds	5º C below T_m of primers
Extension	1 min/kb	72º C
Final extension	10 min	72º C

Table 2-2 Composition of buffers and media

Buffer/Media	Composition	pH
Phosphate-buffered saline (PBS)	137 mM NaCl, 2mM KCl, 10 mM sodium hydrogen phosphate (dibasic), 2 mM potassium hydrogen phosphate (dibasic),	7.4
Tris-EDTA buffer (TE)	10 mM Tris.Cl, 1mM EDTA,	8.0
EB buffer	10 mM Tris.Cl, pH	8.5
Tris-acetate EDTA buffer (TAE)	40 mM Tris, 20 mM sodium acetate, 1 mM EDTA	7.8
Transformation buffer (TFB)-I	30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM magnesium chloride, 15% glycerol, acetic acid to desired pH	5.5
TFB-II	10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% glycerol, KOH to desired pH	6.5
Luria Bertani agar	LB broth plus bacto-agar 15 g/L	7.5
Luria Bertani broth	1% bacto-tryptone, 0.5% bacto-yeast extract, 10% NaCl, pH 7.0	7.5
6X gel loading buffer	0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water	6.8

2.1.3 Restriction digestions and ligations

Restriction digestions for DNA analysis were incubated for at least 1 hour at 37^{o} C in a final volume of 10 µL comprising 1 µL of each restriction enzyme (Promega, Madison, WI or New England Biolabs, Ipswich, MA), 1 µL buffer 10X, 2 µL DNA, 5 µL water. For isolations of backbone or inserts for ligation, digestions were performed in a total volume of 30 µL including 10 µL DNA.

Ligations were incubated overnight at 17º C in the presence of T4 DNA ligase and ligation buffer (New England Biolabs, Hitchin, UK) in a final volume of 10 µL (4 µL of each DNA fragment, 1 µL T4 DNA ligase, 1 µL buffer 10X).

2.1.4 Agarose gel electrophoresis

DNA was electrophoresed in 1% agarose gels (Invitrogen, Carlsbad, CA) with 5 µg/mL ethidium bromide (Dutscher Scientific, Essex, UK) with a 1-Kb Plus DNA size reference ladder (Invitrogen, Carlsbad, CA). For DNA purification, DNA fragments were excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.1.5 Preparation and transformation of competent bacteria

For transformation, 100 µL competent bacteria (prepared from XL1-Blue *E. coli*, Agilent, Santa Clara, CA, using the rubidium chloride buffers TBF-I and TBF-II) were thawed on ice and inoculated with 1 µg of plasmid DNA or 3 µL of ligation reaction. After incubation on ice for 20 minutes, the bacteria were heat-shocked for 2 minutes at 37º C and returned to ice for a further 2 minutes. Transformed cells (ampicillin resistant) were selected in LB agar and ampicillin plates overnight at 37º C.

2.1.6 DNA purification and quantification

Colonies were picked from LB agar plates and grown at 37º C overnight in 4 mL (minipreps), 200 mL (midipreps) or 400 mL (maxipreps) of LB broth with ampicillin (50 µg/mL). Plasmid DNA was purified with QiaPrep Spin Miniprep, Midi and Maxi kits

(Qiagen) following the manufacturer's instructions. DNA concentrations were determined using a Nanodrop 3300 spectrophotometer (Thermoscientific, Wilmington, DE). DNA sequences were verified using standard or customised primers at the University College London Sequencing service.

2.1.7 Western Blotting

Reagents and antibodies are shown in Table 2-3 and Table 2-4. Cells were lysed with 1% Igepal (Sigma-Aldrich) solution and re-suspended in LaemmLi buffer before heating at 96 º C for 5 minutes. Protein concentrations were measured using a bicinchononic acid (BCA) colourimetric assay (Pierce BCA protein assay, ThermoScientific, Rockford, IL). After equilibration for concentration, proteins were separated by SDS-PAGE electrophoresis in a 4% polyacrylamide stacking gel followed by an 11% polyacrylamide separation gel, in SDS running buffer at 150 volts. Proteins were transferred onto Hybond ECL nitrocellulose membrane (Amersham GE Healthcare, Buckinghamshire, UK) using a semi-dry technique (Biorad, Hercules, CA) in transfer buffer at 300 mA for 45 minutes. After incubating for 1hr in blocking buffer at room temperature, membranes were probed with primary antibody (anti-vFLIP 1:400 in blocking biffer, anti-NP, An108, Hytest, Turku, Finland 1:300) at 4 ºC overnight. After washing three times with PBS plus 0.1% Tween 20, anti-mouse (or anti-rat) horseradish-peroxidase (HRP)-conjugated antibody (DakoCytomation, Glostrup, Denmark) was added diluted 1:3000 in blocking buffer. After three washes with PBS plus 0.1% Tween 20, blots were developed with ECL substrate reagents and Hyperfilm ECL (GE Healthcare, Hatfield, UK).

Table 2-3 Western blot buffers and gel reagents

Buffer/gel	Composition
Laemmli buffer	2% sodium duodecylsulphate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.2 mg/mL bromophenol blue, 0.1 M DTT 50 mM Tris (pH 6.8)
11% polyacrylamide gel	11% acrylamide/bis, 125 mM Tris.HCl (pH 8.8), 10% SDS, 0.1% TEMED, 1% ammonium persulphate (APS)
4% stacking gel	4% acrylamide/bis, 125 mM Tris.HCl (pH 6.8), 10% SDS, 0.1% TEMED, 1% APS
Running buffer	25 mM Tris (pH 8.5), 200 mM glycine, 0.1% SDS
Transfer buffer	100 mM Tris, 200 mM glycine, 20% methanol
Blocking buffer	5% semi-skimmed milk, 0.1% Tween 20 in PBS

Table 2-4 Primary antibodies used in western blots

Target	Concentration in blocking buffer	Clone and Maufacturer
Influenza A NP	1:400	An108, Hytest, Turku, Finland
vFLIP	1:400	Made in Collins lab[311]
HBc	1:200	14e11, Abcam, Cambridge, UK

2.1.8 Nuclear RelA translocation assay

To confirm that vFLIP expressed in LV activates NFkB in an IKK dependent manner, a quantitative confocal microscopy assay, previously developed in the Collins laboratory, was used[312]. 4×10^5 pre-B cells (see Cell Lines, below) in 2 mL of IMDM (Gibco) with 50 µM 2-mercaptoethanol were transduced with 10 ng RT vFLIP-GFP or Null-GFP to achieve 30-40 % transduction. The cells were washed on day 1, and on day 2 cells were resuspended in 200 µL Hank's balanced salt solution (HBSS). 100 µL was applied to a poly-L-Lysine coated coverslip (N°1.5, VWR, Lutterworth, UK) and incubated for 15-30min at 37 ºC. Non-adherent cells were removed and cells fixed with 4% paraformaldehyde (Sigma) and stored at 4 ºC before staining. Cells were permeabilised

by application of 50 µL 0f 0.2% Triton X in Tris-buffered saline (TBS)(Sigma) for 10 minutes. After washing in TBS, cells were blocked with TBS 10% goat serum (Sigma) 0.1% azide for 30 minutes at room temperature. Anti-p65/RelA antibody (clone C-20, Santa Cruz Biotechnology) diluted 1:100 in TBS was applied to each coverslip in 50 µL and incubated at 4 ºC overnight. Coverslips were washed with TBS and secondary antibody (Alexa Fluor® 633-conjugated F(ab')2 goat anti-rabbit IgG (Invitrogen)) applied at 4 µg/mL in 50 µL for 1 hour at room temperature. The next day, cells were re-suspended in 3 mL of medium. After washing, coverslips were mounted on glass slides (VWR) using mount solution with DAPI (Sigma) and dried for 3 hours.

A Leica SP2 confocal microscope was used to capture fluorescent images. DAPI, GFP and Alexa Fluor AF633 fluorescence was captured using sequential acquisition to give separate image files for each frequency, using a pin hole of 1 Airy (114.5 µm), 400 Hz scan speed and four-frame averaging. Using photomultiplier tube gain and offset a subsaturating fluorescence intensity (based on DAPI signal) with an optimal signal:noise ratio was selected. ImageJ software (http://rsb.info.nih.gov/ij) was used to create image masks of GFP-, AF633- and DAPI- positive staining. To achieve this 3x3 pixel radius median filter was applied, followed by automatic thresholding (using the IsoData algorithm) for conversion to a binary image. The DAPI staining mask was used to define the nuclear region, whilst subtraction of the of the DAPI mask from the AF633 image defined the cytoplasmic region. A GFP mask was used to identify transduced cells. Each of these staining masks was then applied to AF633 images to measure RelA subunit intensity in the nuclei and cytoplasm of transduced and untransduced cells within each high-power field. Quantitative fluorescence data were exported into Graphpad Prism 5 software for further analysis. Nuclear:cytoplasmic ratios of RelA subunit staining were then calculated by comparison of median values from total GFP-negative and GFP-positive cells.

2.2 Lentiviral vector cloning, production and titration

2.2.1 Lentiviral transfer plasmids

In these experiments the pDual promoter LV backbone, previously described in vaccination experiments by this group, was used[162,313–315]. This incorporates two inserts drive by the spleen focus-forming virus (SFFV) and ubiquitin (UBI) promoters. Early experiments confirmed weak expression of GFP from the UBI promoter in human DC as reported by others[316]. This was therefore replaced with the phosphoglycerate kinase promoter (PGK)[317] for human *in vitro* experiments. Antigen was expressed from the ubiquitin promoter (mouse experiments) and PGK promoter (human experiments), whilst molecular adjuvants (vFLIP or 4-1BBL variants) were expressed from the SFFV promoter. In experiments where antigen-only expressing LV were required, molecular adjuvants at the first promoter were replaced by a 6 base-pair non-coding sequence (ACTAGT), designated “null”.

cDNA sources: vFLIP was derived from pDual-vFLIP-iOVA, previously described by Dr Helen Rowe[314]. A pGMT plasmid encoding influenza nucleoprotein derived by reverse transcription from X31 virus (identical in sequence to NP from A/PR/8/34) was kindly provided by Dr Claire Bennett. In human experiments, adw strain Hbc was used as a control for non-antigen specific T cell responses (provided by Dr Mike Whelan (iQur Ltd)). 4-1BBL (human and mouse) cDNAs were sourced from Source Bioscience, Nottingham, UK. E coli expressing plasmids 4-1BBLH (IRCKp5014E0818Q) and 4-1BBLM (IRATp970d03124D) were supplied as stab cultures. cDNA were amplified using the primers shown below.

Forward and reverse primers were used to amplify cDNA template plasmids with incorporated 5’ BamH1 and Kozak sequence (Forward primer) and 3’ Not1 (reverse primer) for insertion into pGMT and (following verification sequencing to exclude mutations) subsequent digestion and ligation into the LV backbone at the first insertion site. For insertion into the second site, PCR primers incorporating KpnI or MLuI (5’) and Xho1 (3’) were used.

The truncated version of 4-1BBL(mouse) was created using a forward primer containing (5' to 3') a BamH1 restriction site sequence, Kosack sequence, start codon and a 15bp sequence homologous to base pairs 234-251 of the *mus musculus* 4-1BBL cDNA. This region encodes for 5 amino acids proximal to the transmembrane region of 4-1BBL. Reverse primers used were identical to those used to amplify 4-1BBL WT.

In order to test the principle that LV transgene expression could be selective silenced in AM, a transfer vector was constructed in which a marker transgene cDNA (4-1BBL) was continuous with 4 repeats of a target sequence for the haematopoetic-specific microRNA, miR-142-3p as previously described[318]. A template plasmid containing this sequence was kindly provided by Dr David Escors (created using overlapping oligonucleotides as described by Annoni *et al*[150]) and amplified by PCR using primers incorporating Not1 (forward) and SBf1) (reverse) in order to digest and insert the sequence after the first transgene in the dual promoter backbone.

Sequences of PCR primers used are shown in Table 2-5.

Table 2-5. Sequences of oligonucleotides used for PCR amplification of target sequences

Name	Sequence
BamH1-vFLIP-Fw	GGATCCGCCACCATGGCCACTTACGAGG
Not1-vFLIP-RV	GCGGCCGCCTATGGTGTATGGCGATAGT
Kpn1-NP-Fw	TGGTACCGCCACCATGGCGTCCCAAGGC
Mlu1-NP-Fw	ACGCGTGCCACCATGGCGTCCCAAGGCA
Xho1-NP-Rv	CTCGAGTTAATTGTCGTACTCCTCTGCATTG
EcoR1-PGK-Fw	GAATTCCCACGGGGTTGGGGTTGCGCCT
Mlu1-PGK-Rv	ACGCGTCCTGGGGAGAGAGGTCGGTGATTCG
BamH1-4-1BBLTc-Fw	GGATCCGCCACCATGCGCCACCCAAAGCTC
BamH1-4-1BBLm-Fw	GGATCCGCCACCATGGACCAGCACACAC
Not1-4-1BBLm-Rv	GCGGCCGCTCATTCCCATGGGTTGTCGG
BamH1-4-1BBLH-Fw	GGATCCGCCACCATGGAATACGCCTCTG
Not1-4-1BBLH-Rv	GCGGCCGCTTATTCCGACCTCGGTGAAG
Mlu1-HBc-Fw	ACGCGTGCCACCATGGACATTGACCCTT
Xho1-HBc-Rv	CTCGAGCTAACATTGAGATTCCCGAGAT
Kpn1-GFP-Fw	GGTACCACCGGTCGCCACCATGGTGAGCAA
Xho1-GFP-Rv	CTCGAGTTTACTTGTACAGCTCGTCCAT
Sma1-m1423p-Fw	CCCGGGGGCCGCCGACTCCATAAAGT
Sbf1-m1423p-Rv	CCTGCAGGTCGACTCTAGTGTAGTGTTT

2.2.2 Lentiviral vector production

LV were produced by a 3 plasmid transient transfection of 293T cells using a a transfer vector, an HIV-1 derived packaging plasmid (p8.91) and a plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) (pMD.G), as described previously[124] and in the introduction. p8.91 and pMD.G were produced by Plasmid Factory (Bielefeld, Germany).

10^7 293T cells were plated in 14 cm plates to reach 80-90% confluence the following day. Fugene 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) was used to enhance transfection using the following mix (per plate):

Reagent	Quantity
p8.91	2.5 µg
pMD.G	2.5 µg
Transfer plasmid	37.5 µg
Optimem (Gibco)	500 µL
Fugene 6	45 µL

The transfection mix was added dropwise to each plate and the medium changed 9 hours later. The supernatants were collected at 48 and 72 hours and passed through a 45 µm filter and stored at 4 C until concentration by centrifugation.

LV particles were concentrated 200-fold in 35 mL ultracentrifugation tubes (Beckmann-coulter) by two rounds of ultracentrifugation (115 000 x *g* for 2 hours at 4 ºC) in a Sorvall ultracentrifuge. UC tubes were filled with a 5 mL 20% sucrose cushion overlaid with 30 mL supernatant. Viral pellets were re-suspended in HBSS 10% glycerol and frozen at -80º C until use.

2.2.3 LV titration

FACS titration

FACS titration measures numbers of transcriptionally active, integrated transgene cassettes and thus encompasses efficiency of transduction as well as gene expression. It is thus sensitive to cell-specific factors such as the availability of transcription factors for a chosen promoter.

To titrate GFP-encoding LV, 2 x 10^5 293T cells were transduced with 25 µL of serial 1:5 dilutions of LV stock in 12-well plates. After 3 days, the percentage of GFP-expressing cells was determined by FACS. Infectious units per mL were determined from a sample in the non-saturated (linear) portion of the saturation curve as follows:

Viral titre (IU/mL) = 2 x 10^5 cells x % transduction x (dilution factor / 25 µL) x 1000

Reverse transcriptase quantification

Quantification of RT activity in viral stocks gives an indirect measure of number of viral particles, but does not determine the number of functional infectious particles (since "empty" particles lacking RNA may be RT active).

RT activity was measured using a Reverse Transcriptase Assay colorimetric kit (Roche), according to the manufacturer's instructions. Concentrated virus was diluted 1:20-1:100 and incubated for 3 h for the RT reaction.

2.3 Tissue Culture

2.3.1 Cell lines

293T cells are a hypotriploid immortalised line derived from human embryonic kidney cells. They are readily transfected (and transduced by LV) and demonstrate high expression of transgenes[319] making them ideal for both LV packaging and as target cells to test LV transgene expression. 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Paisley, UK), with 10% Foetal Calf Serum (FCS) (Serotec, Oxford, UK), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco) and 2 mM L-glutamine (Gibco). Cells were passaged 1:4 using trypsin/EDTA (Gibco) every 3 days.

MDCK cells were used for titration of influenza virus (see below). These were cultured in DMEM with 10% FCS and passaged 1:4 using trypsin/EDTA every 3 days.

Pre-B cells were used for the relA translocation assay. These were cultured in Roswell Park Memorial Institute (RPMI) Medium (Gibco) with 10% FCS and passaged 1:8 every 3 days. An IKKγ-deficient mouse pre-B cell line, 1.3E2, derived from wild-type (WT) 70Z/3 cells, together with 1.3E2 cells reconstituted with WT IKKγ were kindly provided by Dr Akira Shiamzu, as described previously[320].

2.3.2 Mouse bone marrow-derived dendritic cells

Bone marrow-derived DC were extracted using a method modified from Talmor *et al*[321]. The bone marrow of BALB/c mice was flushed from the femur and tibia with Hank's buffered salt solution (HBSS, Gibco) supplemented with 1% FCS using a 25G needle and 5 mL syringe. Red blood cells were lysed with re-suspension in red cell lysis buffer ((Sigma) for 5 minutes before washing twice in HBSS 1% FCS an then re-suspending at 5-7.5 x 10^5 cells/mL in RPMI supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol (Gibco) and 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ). On day 4 non-adherent DC were harvested for use in transduction experiments and then cultured in the same media thereafter. 10 ng/RT of LV typically gave 50% transduction of 500,000 murine DC as measured by FACS expression of GFP.

2.3.3 Human monocyte-derived dendritic cells

60 mL of blood from healthy volunteers were diluted 1:2 in RPMI and peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation using Ficoll Paque PLUS (GE Heatlhcare Life Sciences, Buckinghamshire, UK). CD14+ cells were isolated by positive selection using a magnetic-activated cell sorting system (MACS) according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). 60 mL of whole blood typically yielded 10 million CD14+ cells which were cultured at a density of 1x10^6 cells/mL in an upright flask in X-vivo 15 Media (Lonza, Walkersville, MD)

supplemented with 1% human AB serum, (human GM-CSF (Peprotech) and human IL-4 (Peprotech) both at 50 ng/mL.

2.3.4 Isolation of CD14- human peripheral blood mononuclear cells (PBMC)

Human PBMC were isolated from whole blood by centrifugation over Ficoll (GE Healthcare) as described above. PBMC were frozen at $1x10^7$ cells/mL in FCS 10% DMSO (Sigma) and stored at -80 ºC until use.

2.4 Mouse Vaccination and Influenza challenge

Female BALB/c mice, 6-8 weeks old, were purchased from the UK branch of Charles River Laboratories (Wilmington, MA) and bred in pathogen-free conditions in an animal house facility at the Windeyer building, University College London (and Kathleen Lonsdale building after June 2011).

2.4.1 Vaccination

A dose of 50 ng RT was given for subcutaneous vaccination (given into the hindquarter). This dose was chosen after early titration experiments established a fall in IFNγ+ CD8+ T cell responses against NP by ELIspot below 25 ng RT.

Intranasal vaccination was performed under ketamine and xylazine anaesthesia (20 mg/Kg and 2 mg/Kg respectively) by inoculation of 20 µL of viral suspension into each nostril. A dose of 200 ng RT per mouse was used, derived from previously published data using the same VSV-G pseudotyped LV in mouse models of intranasal LV delivery for gene therapy[322]. 20 µL into each nostril ensured aspiration into distal lung and transient tachypnoea lasting 10 minutes.

2.4.2 Influenza strains and challenge

Intranasal challenges with influenza virus were performed in the same way as intranasal vaccination. Following challenge, mice were weighed daily from day 3 onwards. Mice were sacrificed if weight loss exceeded 25% under the terms of the corresponding Home Office license. Influenza strains used were mouse-adapted A/PR/8/34 (a gift from Dr Mike Whelan, iQur Ltd) and A/Eng/195/09 an H1N1 strain from the recent SO H1N1 pandemic that was isolated from a subject with mild influenza symptoms (a gift from Prof Wendy Barclay, Imperial). LD_{50} titration was determined by infecting groups of 7 mice with incremental doses of PR8 from 100 – 10,000 PFU per mouse. A lethal dose of $2xLD_{50}$ corresponded to 2500 PFU per mouse by plaque assay. A 0.8 xLD_{50} dose was given to unvaccinated mice in experiments investigating the primary T cell response to ensure survival of some mice for the 15 day duration of the experiment. A/Eng/195/09 is non-lethal in mice even at high doses. A/Eng/195/09 challenges used $5x10^4$ PFU per mouse which was the minimum dose that resulted in 10% weight loss in control mice.

2.4.3 Plaque assay

Influenza virus in stocks and lung lysates were titrated by plaque assay (reagents shown in Table 2-6). MDCK cells were seeded in 12-well plates to form a confluent monolayer and incubated overnight. After washing cells in PBS, virus stock/lung lysate was added after serial 10-fold dilutions. Plates were incubated at 33 ºC for 1 hour. 17.5 mL of Flu overlay (per 12-well plate, see reagent list) was warmed to 37 ºC. 2% plaque agarose melted in a 55 ºC water bath and 7.5 mL added to Flu Overlay together with 50 µL Trypsin. The inoculum was removed from each well and 1 mL overlay added. After agarose had set, plates were inverted and incubated for 3 days to allow plaques to form. Plaques were counted after removal of agarose and addition of crystal violet stain for 10 minutes.

Table 2-6 Reagents for influenza plaque assay

Buffer/gel	Composition/Manufacturer
Flu Overlay	100 ml 10x MEM (GIBCO), 28 mL 7.5 % BSA (fraction V, GIBCO), 10 mL L-Glutamine , 20 mL 7.5 $NaHCO_2$, 10 ml 1M HEPES, 5ml 1% dextran, 10 mL Penicillin/Streptomycin, 517 mL H_20
Trypsin	1 mg/mL (Worhtington, Reading, UK)
2% Agarose	6g Agarose (Oxoid, Basingstoke, UK) in 200 mL H_20
Crystal violet stain	40ml 1% Crystal violet in water (Sigma) , 80ml ethanol, 300ml H_20

2.5 Immunoanalysis

2.5.1 Cell retrieval

Splenocytes

Splenocytes were dissected from exsanguinated mice and mashed through 70 µM pore strainers (VWR) in 2 mL of HBSS 1% FCS. Splenocytes were washed in HBSS 1% FCS and re-suspended in red-cell lysis buffer. Following two further washes splenocytes were counted and then either stained and analysed by FACS or stimulated overnight with peptide for analysis by ELISpot or intracellular cytokine staining the following day.

Lymph nodes

Lymph nodes were dissected from the inguinal region of exsanguinated mice and mashed through 70 µM pore strainers (VWR) in 2mL of HBSS 1% FCS. Following two washes cells were counted and analysed by FACS or stimulated overnight with peptide for analysis by Elispot or intracellular cytokine staining the following day.

Broncho-alveolar lavage

Mice were dissected and the trachea exposed. Broncho-alveolar lavage (BAL) was performed by instillation and retrieval of 2 mL of HBSS through a 16G Venflon® catheter (BD) into the transected trachea. Samples were centrifuged and supernatants stored for cytokine analysis. Cells were washed in HBSS and re-suspended in 200 µL FACS buffer for staining or media for culture.

Total lung

Lungs were exsanguinated by cardiac puncture and lymph nodes dissected away after removal. After weighing, lungs were mashed through 70 µM pore strainers (VWR) into 2 mL of HBSS supplemented with 1% FCS. Cell suspensions were layered onto 1.5 mL Histopaque® 1083 (Sigma) in 15 mL v-bottomed centrifuge tubes (BD Falcon) and centrifuged at 800g in a Sorvall RT legend centrifuge at room temperature with no brake. The buffy coat was aspirated and washed twice in media-free RPMI before re-suspending in RPMI 10% FCS for culture or in PBS for immediate FACS staining.

Blood

Blood was obtained by cardiac puncture (into heparinised tubes) from mice under non-recovery anaesthesia. Samples were diluted to a total volume of 2 mL with serum-free RPMI and separated over 1.5 mL Histopaque 1083 (Sigma) and processed there forth in the same manner as lung samples.

2.5.2 Enzyme-linked immunospot assay

Enzyme-linked immunospot assay (ELISpot) plates (Millipore, Billerica, MA) were covered at 4 ºC overnight with 5 µg/mL of purified anti-IFNγ (BD Pharmingen, San Diego, CA). The following day, plates were washed and blocked for 2 hours with RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Splenocytes were re-suspended in RPMI and 1 x 10^6 added per well. The plate was incubated for 20 hours at 37 ºC, in the presence of class I or class II peptide (see Table 2-8).Cells were lysed with water and plates washed with PBS. Plate-bound IFNγ was determined with serial labelling with 0.5 mg/mL biotinylated anti-IFNγ antibody (BD Pharmingen), 1:10000 streptavidin-conjugated alkaline phosphatase (Caltag, Burlington CA) and then an Alkaline Phosphatase Conjugate Substrate Kit (BioRad, Hercules, CA). An AID ELISPOT counter and software was used to count spots.

2.5.3 Antibody labelling for FACS

All stains were performed in round-bottomed 96-well plates (Helena Biosciences, Gateshead, UK). Primary antibody panels used are shown in Table 2-7. If applicable, cells were first incubated with 5 µL $NP_{147-155}$ pentamer-PE in 30 µL PBS (ProImmune, Oxford, UK) for 15 minutes at room temperature. Cells were then washed by addition of 200 µL PBS per well and centrifugation at 400g for 5 minutes. All subsequent staining steps were performed in the presence of a blocking antibody mix consisting of rabbit serum, goat serum and rat anti-Fc antibodies. All primary antibodies were diluted in PBS (containing blocking mix at a 1:50 dilution) at the specified concentration and 50 µL added per well before incubation for 1 hour at 4º C. After washing twice with PBS, secondary antibody was added (if applicable) and cells re-incubated. Otherwise cells were permeabilised using FoxP3 fixation/permeabilisation solution (eBioscience, Hatfield, UK) for 20 minutes. Subsequent antibody labelling of internal targets was performed in permeabilisation buffer (eBioscience) supplemented with Blocking solution at a dilution of 1:50. Permeabilised cells were incubated with

antibody for 1 hour before washing and re-suspending in permeabilisation buffer prior to analysis.

All samples were analysed on a BD FACS Calibur (before 2009) or BD LSR 2 or BD Fortessa (post-2009), using Cellquest or FACSDiva software respectively. FACS gating strategies are shown in Figure 2-1.

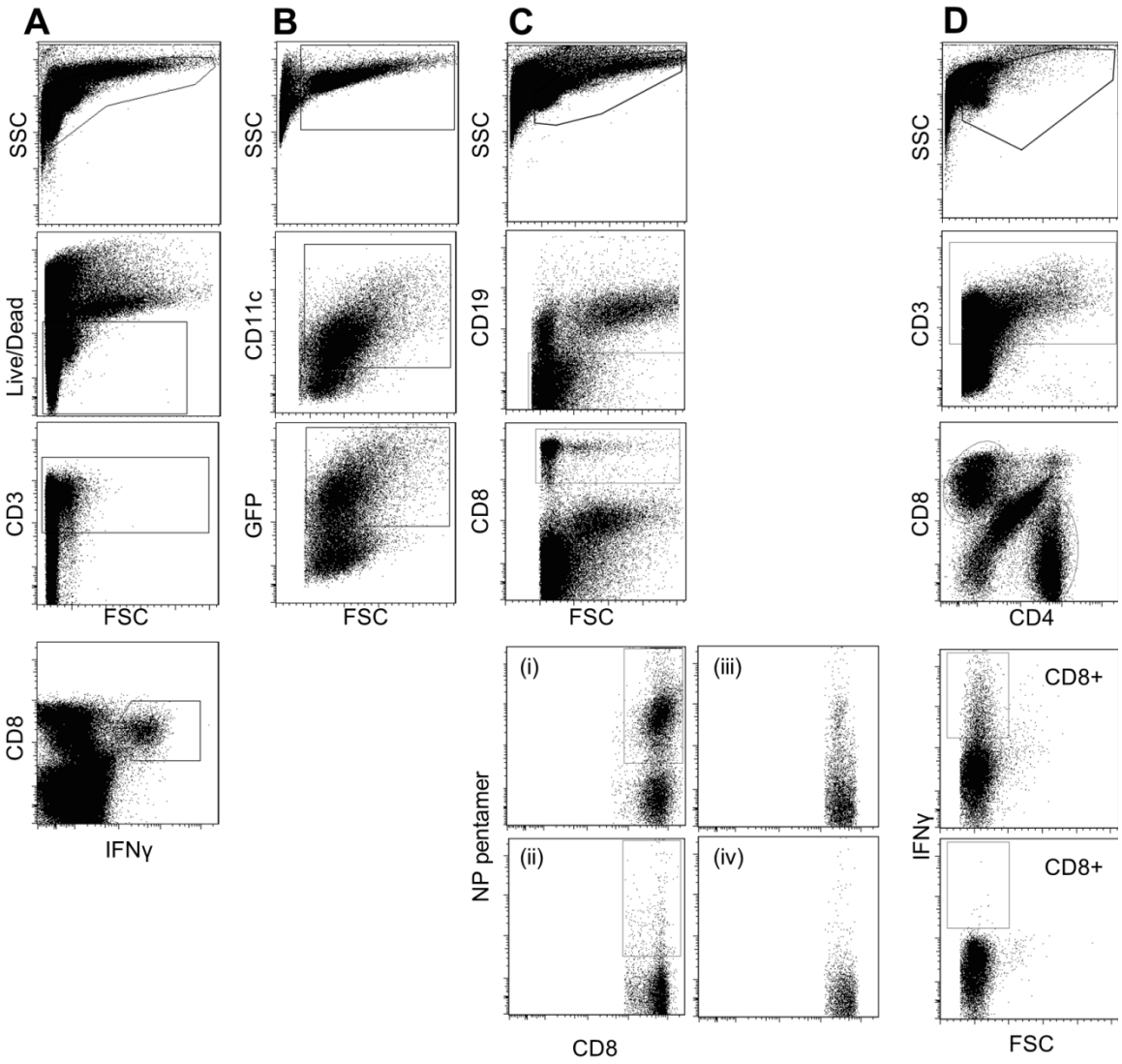


Figure 2-1 FACS gating strategies. **(A)** Analysis of human PBMC following 10-day expansion with autologous LV-transduced DC and overnight NP peptide stimulation. Example shows IFNγ staining after expansion with DC transduced with vFLIP-NP (see Figure 9C) **(B)** Gating of LV-transduced BALB/c DC by GFP positivity (see Figure 3A) **(C)** Quantification of CD19-, CD8+, $NP_{147-155}$ pentamer+ T cells in lung homogenate (See Figure 4) in (i) SC-IN vFLIP-NP immunised mouse (ii) unimmunised control (iii) splenocytes gated in the same way stained with $NP_{147-155}$ pentamer and (iv) irrelevant OVA pentamer **(D)** Intracellular cytokine staining in T cells from lung homogenate. Example shown is IFNγ staining of CD8 T cells from lung homogenate day 6 post lethal influenza challenge in vFLIP-NP SCx2 immunised mice after overnight NP peptide re-stimulation (i) and unstimulated (ii).

Table 2-7 FACS antibody panels

Panel		Anti-Mouse Antibody	Clone	Company	Dilution
Pentamer quantification with homing phenotype	Extracellular	CD19-AlexaFluor®700	1D3	eBioscience	1:200
		$NP_{147-155}$ Pentamer	F098	Proimmune	5 µL/well
		CD8-APC	53-6.7	eBioscience	1:200
		CD62L-eFluor® 450	MEL-14	eBioscience	1:200
		CD127-FITC	A7R34	eBioscience	1:200
Pentamer cytotoxicity/ proliferation	Extracellular	$NP_{147-155}$ Pentamer	F098	Proimmune	1:6
		Fixable viability dye-eFluor® 780	65-0865-18	eBioscience	1:1000
		CD3-PERCP-Cy5.5	17A2	Biolegend	1:200
		CD4-v500	RM4-5	BD	1:100
		CD8-v450	53-6.7	ebioscience	1:150
	Intracellular	GzmB-APC	GB11	Invitrogen	1:75
		Ki67-FITC	20Raj1	ebioscience	1:50
		FOXP3-AlexaFluor®700	FJK-16s	eBioscience	1:500
T cell post-re-stimulation	Extracellular	Fixable viability dye-eFluor® 780	65-0865-18	eBioscience	1:1000
		CD3-PERCP-Cy5.5	17A2	Biolegend	1:200
		CD4-v500	RM4-5	BD	1:100
		CD8-v450	53-6.7	ebioscience	1:150
	Intracellular	GzmB-APC	GB11	Invitrogen	1:75
		IFNγ-FITC	XMG1.2	eBioscience	1:75
		TNFα-PE	MP6-XT22	BD	1:75
		IL-2-PE-Cy7	JES6-5H4	eBioscience	1:75

DC Activation (sample split for 6 separate stains)	Extracellular	CD11c-APC	N418	eBioscience	1:300
		CD80-PE	1610A1	eBioscience	1:200
		CD86-PE	PO3.1	eBioscience	1:200
		ICAM1-PE	YN1/1.7.4	eBioscience	1:200
		CD40-PE	1C10	eBioscience	1:200
		MHCII-PE	NIMR-4	eBioscience	1:200
		PDL1-PE	MIH5	eBioscience	1:200
DC 4-1BB and 4-1BBL	Extracellular	CD11c-APC	N418	eBioscience	1:300
		4-1BB-PE	17-B5	eBioscience	1:250
		4-1BBL-Biotin + streptavidin-eFluor450	TKS-1	eBioscience	1:250
Alveolar macrophage	Extracellular	CD11c-APC	N418	eBioscience	1:300
		F4/80-eFluor450	BM8	eBioscience	1:200
		CD86-PE	PO3.1	eBioscience	1:200
		CD80-Biotin + streptavidin-eFluor605NC	1610A1	eBioscience	1:200

Panel		Anti-Human Antibody	Clone	Company	Dilution
T Cell re-stimulation	Extracellular	Live/Dead fixable stain Pacific Blue	L34957*	Invitrogen	1:1000
		CD4-APCCy7	OKT-4	eBioscience	1:200
		CD3-PeCy7	UCHT1	eBioscience	1:300
		CD8-AlexaFluor700	OKT-8	eBioscience	1:200
	Intracellular	TNFα-PE	MP6-XT22	eBioscience	1:50
		IFNγ-APC	4S.B3	eBioscience	1:80
		IL2-FITC	MQ1-7H12	eBiosceince	1:70

Table 2-8 Peptides used for T cell re-stimulation

HLA restriction	Restriction	Sequence
$NP_{147-155}$	H2-K^d class I	TYQRTRALV
NP_{55-78}	H2-K^d class II	RLIQNSLTIERMVLSAFDERRNKY
$NP_{265-274}$	HLA-A3 class I	ILRGSVAHK
$HBc_{18-27\ (control)}$	HLA-A2 class I	FLPSDFFPSV

2.5.4 Cytometric Bead Array

In order to measure cytokines in cell culture supernatant, BAL or lung lysate cytometric bead arrays (BD) were used according to the manufacturer's instructions. These kits use antibodies against specific cytokines bound to two groups of differently-sized beads. Each group contains beads of 5 separate fluorescence intensities, each specific for a cytokine. Analyte bound to a bead is detected by a second antibody (PE-conjugated) and the PE signal is proportionate to the amount of bound analyte. A standard curve is generated with provided cytokines of known concentration. All samples were analysed in 96 well plates in a BD FACSArray™ bioanalyser. Cytokine panels are shown in Table 2-9 below.

Table 2-9. Cytometric bead array panels

Panel	**Cytokine (mouse)**
TH-1/TH-2/TH-17	IL-1alpha, IL-2, IL-5 IL-6, IL-10, TNFα IFNγ, IL-17, GM-CSF IL-4
T cell chemoattractant	RANTES, MIP-1α MIP-1β, IP-10 MCP-1, MCP-3

2.5.5 AM depletion

Depletion of AM was attempted (see results page 162) with clodronate liposomes (ordered from clodronateliposomes.org). 40 µL of 5 mg/mL liposome suspension (clodronate or PBS control) was administered intranasally to anaesthetised mice as described above. The procedure was repeated 2 days later. 4 days after the second administration, mice were euthanized and BAL performed. AM were stained and quantified by FACS as described above.

Depletion of AM was also attempted by intranasal administration of diphtheria toxin to CD11c-diptheria toxin receptor (DTR) transgenic mice as described by van Rijt *et al*[323]. CD11c-DTR BL/6 transgenic mice were a kind gift from Dr Claire Bennett. The mice express the diphtheria toxin receptor driven by the CD11c promoter, resulting in expression in dendritic cells and alveolar macrophages. This permits selective killing of these cell types with *in vivo* administration of diphtheria toxin. 100 ng DT (in 40 µL) was administered to anaesthetised mice intranasally. AM numbers were assessed by sacrifice and BAL 4 days later.

2.5.6 Adoptive transfer of AM

AMs were transduced *in vivo* by intranasal administration of LV at 200 ng RT per mouse. 2 days later, mice were sacrificed and 2x2 mL BAL performed on each mouse. Cells were pooled within vaccination groups, washed and re-suspended in HBSS, and transferred intranasally into recipients. 1.5 donor mice were used per recipient. 40 µL was left aside from each group for FACS quantification of the total number of AM transferred after staining for F4/80 and CD11c. 2 weeks after adoptive transfer mice were either sacrificed for analysis (n=3) or challenged with lethal A/PR/8/24 influenza (n=5). In initial experiments attempted purification of AM by adherence substantially reduced the numbers that could be harvested for transfer. However, the high specificity of AM transduction (>99%) by intranasal LV permitted transfer all cells from the BAL without sorting or purification. Sufficient AM could be harvested from 5 mice to transfer between 10,000-13000 F4/80+CD11c+ cells to each of 3 recipient mice.

2.5.7 Human PBMC re-stimulation with autologous LV-transduced monocyte-derived DC

Human CD14+ cells were isolated from peripheral blood mononuclear cells (PBMCs) and cultured as described above. On day 4, non-adherent cells were harvested and transduced with LV at 22 ng RT per 100,000 DC in the presence of protamine 10 µg/mL at a concentration of $1x10^6$ cells/mL. Control DC were matured with IFNγ 100 IU/mL and LPS 100 ng/mL and pulsed with either HLA-matched NP class-I peptide or hepatitis B core peptide. Cells were incubated for 3 days in 96-well plates before washing and re-suspension in media with addition of thawed CD14- autologous PBMCs. DC and PBMC were co-cultured for 10 days, replenishing 50% of the media at day 5. Prior to FACS analysis, cells were re-stimulated overnight with the appropriate HLA-matched NP peptide in the presence of Brefeldin A 1 µg/mL. PBMC were stained according to the antibody panels shown in Table 2-7.

2.6 Statistical analyses

All data were analysed using the GraphPad Prism v5.0 statistical software package. Statistical tests applied to each data set are indicated in the relevant figure legend.

3 Systemic vaccination against influenza with lentiviral vectors

3.1 Introduction

3.1.1 DC activation for improved T cell responses to vaccination

DC are potent antigen presenting cells - as few as 300 antigen-pulsed DC are required to generate proliferative responses in several million T cells[324]. Exploiting this ability to stimulate T cell responses against infection and cancer has been a major focus of immunotherapy research for several decades. A key early finding in such work was that for effective T cell priming, DC had to both present antigen and also undergo activation. Activated DC express the co-stimulatory molecules and cytokine signals necessary to prime effective T –cell responses (the 3 signal hypothesis). Current opinion is that the pattern of co-stimulatory molecules and cytokine production is determined by the dominant pathway of DC activation, which in turn is directed by the pattern of PRR stimulation by PAMPs or DAMPs. However, there is growing recognition of the role of antigen presenting cells in peripheral tolerance; silencing T cell clones with self-reactivity that have escaped deletion in the thymus in order to avoid autoimmunity. Like immunogenicity, immunotolerance is difficult to generate with antigen alone. DC play a central role under steady- state condition in presenting self-antigens and maintaining tolerance[325,326,] and can be harnessed to enhance the deletion or anergy of T cells specific to a chosen antigen[327]. DC can also be exploited to enhance T regulatory (CD25+Foxp3+) responses and actively suppress autimmunity[328]. Effective vaccines must minimise forms of DC activation that generate co-inhibitory signals and thus tolerance, anergy or deletion, since these may impair the immune response to subsequent infectious challenge. Suppressing a tolerogenic response entirely may be undesirable however, since this is an essential part of resolution of inflammation and limitation of collateral tissue damage.

Stimulating PRRs with synthetic or naturally occurring PAMPS in order to enhance DC co-stimulatory molecule expression and therefore T cell responses is a fundamental tenet of vaccine and adjuvant design. Viral and gene based vectors provide

opportunities to activate DC at multiple levels of signalling between PRR stimulation and up-regulation of co-stimulatory molecule expression (Figure 3-1). These range from co-encoding antigen with a chosen single co-stimulatory molecule, to direct activation of signalling pathways involved in pathways of activation that lead to desirable patterns of co-stimulatory molecule expression. The principle advantage of DNA or viral vector based vaccines – the endogenous production of antigen and efficient class I processing – can thus be supplemented with signals which activate DC in a manner that ultimately gives rise to a desirable effector or memory T cell profile.

Previous work in this laboratory has explored these concepts using lentiviral vectors to deliver both antigen and DC-modifying signals with the purpose of manipulating the resultant T cell response. Other groups have also shown that LV expressing DC activators can enhance T cell responses to antigen. LV expressing GM-CSF and IL-4 have been used to drive monocyte differentiation into DC and improve autologous human CD8+ T cells responses against pulsed antigens[329], whilst LV expressing CD40L and OX40L have been used to induce self-maturation of DC ex vivo[330,331]. LV expressing HSP 70, which activates TLR 2 and 4 in DC, fused to melanoma antigen (tyrosinase-related protein-2, TRP2), delivered subcutaneously generated an IFNγ-producing CD8+ T cell response associated with anti-tumour immunity in a subcutaneous B16 melanoma model in mice[332].

One key advantage of using lentiviral vectors in this role is their low intrinsic immunogenicity minimises vector-mediated DC activation and permits discrimination of the effects of the encoded activator. The integrating nature of LV also permits examination of the long-term consequences of persistent expression of the activator and antigen by APCs.

3.1.2 Activation of DC can be immunogenic or tolerogenic

Transduction of DC with LV expressing activators or dominant negative inhibitors of the signalling pathways (Figure 3-1) can result in diverse patterns of DC activation and similarly varied T cell responses to co-encoded antigen. Two major signalling pathways, the MAPK and NFκB pathways have been consistently implicated in DC activation. A

number of PRRs, both intracellular and membrane-bound, initiate signals which feed into these final common pathways. The cytoplasmic tails of TLRs, for example, recruit two Toll/IL-1 receptor (TIR) domain containing adaptor molecules after ligand engagement: myeloid differentiation factor 88 (Myd88) and TIR domain-containing adaptor-inducing IFN-β (TRIF). These form an essential framework to which protein kinases and ubiquitin kinases bind and activate MAPK and NFκB pathways[333].

MAPKs can be divided into three groups: the p38 stress-activated protein kinases (p38), c-Jun protein kinase (JNK) and extracellular signal regulated kinases (ERK). Activation of MAPKs through phosphorylation requires a three-stage kinase cascade beginning at the membrane (or endosome membrane) level. Different patterns of membrane TLR stimulation combined with other signalling receptors (such as the TNF-receptor family) will result in differential stimulation of the three final MAPK pathways. For example, ERK is activated by TLR recruitment of TNF-receptor associated factor (TRAF) 6 and TRAF3 together with tumour progression locus 2 (Tpl2)[334]. ERK activation decreases co-stimulatory molecule expression and increases secretion of immunosuppressive cytokines such as TGF-β[335] and IL-10[336]. P38 phosphorylation by MAPKKs, MKK3 and MKK6 is dependent upon transforming growth factor B-activated kinase (TAK-1) rather than Tpl2 recruitment to the TRAF6/Myd88 complex. This leads to DC maturation and expression of co-stimulatory molecules such as CD80, CD86, CD83 and CD40[337]. Similarly, JNK is activated by TLR signals via MKK3, MKK4 and MKK7 and results in pro-inflammatory cytokine secretion although its role in DC maturation is less well-characterised[338].

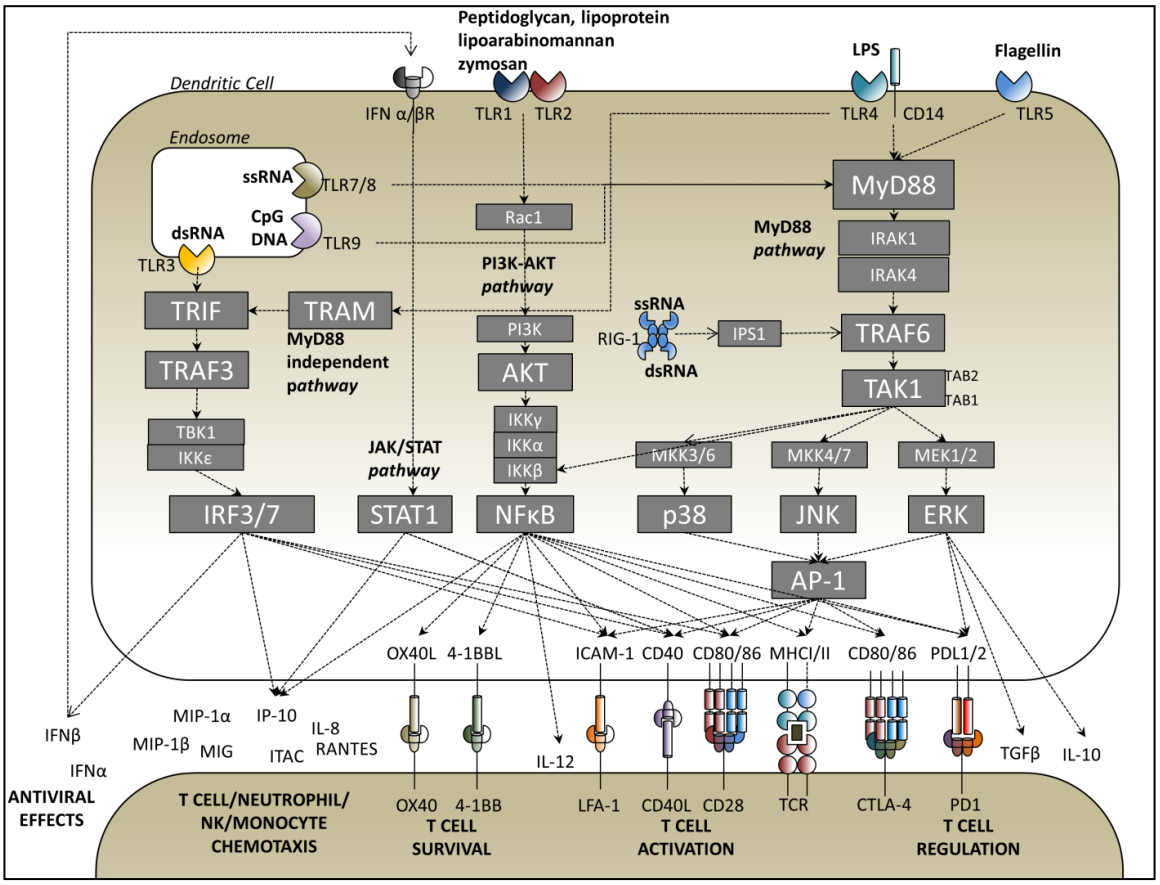


Figure 3-1 Pattern recognition receptor signaling pathways in DC and resulting patterns of co-stimulatory molecule and cytokine expression. **TLR** Toll-like receptor, **LPS** lipopolysaccharide, **Rac1** Ras-related C3 botulinum toxin substrate 1, **PI3K** Phosphatidylinositide 3-kinases, **IKK** inhibitor of nuclear factor kappa-B kinase, **NFκB** nuclear factor kappa-B, **TAK1** Mitogen-activated protein kinase kinase kinase 7, **MKK** mitogen-activated protein kinase kinase, **MyD88** Myeloid differentiation primary response gene (88), **IRAK** Interleukin-1 receptor-associated kinase, **TRAF** TNF-receptor associated factor, **TRAM** TRIF-related adaptor molecule, **TRIF** TIR-domain-containing adapter-inducing interferon-β, **TBK1** TANK-binding kinase-1, **ERK** extracellular signal-regulated kinase, **JNK** c-Jun N-terminal kinase, **STAT1** Signal Transducers and Activators of Transcription 1, **IRF** interferon regulatory transcription factor, **JAK** Janus kinase, **PDL-1** programmed cell death ligand-1, **IFN** interferon, **MIP** macrophage inflammatory protein, **MIG** monokine induced by gamma interferon, **IP-10** Interferon gamma-induced protein 10, **ITAC** Interferon-inducible T cell alpha chemoattractant, **RANTES** Regulated on Activation, Normal T cell Expressed and Secreted.

NFκB is a transcription factor activated in DC by several pro-inflammatory stimuli including TLR ligands and cytokines. NFκB responsive elements are found in a wide range of promoters upstream of genes involved in inflammation[339]. There are two main pathways of activation: classical and alternative. The former is activated through TNF-receptor family signalling, TLRs and intracellular PRRs such as melanoma differentiation associated factor (MDA) 5, retinoic acid inducible gene (RIG) 1 and protein kinase R (PKR)[340]. These activate the main regulator of NFkB signalling, inhibitors of Kappa-B (IκB) Kinase (IKK). This is composed of two catalytic subunits IKKα and IKKβ, and a third regulatory subunit IKKγ, also known as NFkB essential modulator (NEMO). The NFκB transcription factor consists of homo- and hetero-dimeric complexes of 5 subunits: p65/RelA, RelB, c-REl, p50 and p52. The Rel domains bind IκBs in the cytoplasm, preventing translocation of the transcription dimers to the nucleus. IκBs are phosphorylated by IKK, leading to their degradation by ubiquitination and allowing translocation of the p50-RelA heterodimers to the nucleus where they act upon NFκB responsive elements in promoters. In the alternative pathway of activation, IKKα phosphorylates the NFκB subunit precursor p100, leading to release of the p52 fragment which translocates to the nucleus as a heterodimer with RelB. This is initiated by a number of upstream signalling pathways originating from receptors to lymphotoxin-β, CD40L and receptor activator of NFκB ligand (RANKL), which release inhibitory ankyrin repeats in the c-terminus of p100[341].

By overexpressing or using constitutively activated or dominant negative mutants of signalling components of these pathways it is possible to determine their role in generating immunogenic or tolerogenic effector function in dendritic cells. This group has previously activated the p38, ERK and JNK MAPK pathways using a constitutively activated MKK6 (MEKK6), MEK-1 and MKK7-JNK fusion protein respectively. These activators typically contain acidic amino acids such as glutamate and aspartate in place of the serine/threonine residues in their phosphorylation dependent activation loop. Activation of p38 MAPK through transduction with LV expressing a constitutively active MKK6 resulted in partial maturation of DC comparable to LPS, a prototypic TLR-4 stimulator. Specifically, co-stimulatory molecules CD80, CD40 and ICAM-1 were up-regulated but there was no increase in "3rd signal" cytokines such as IL-12[342]. Co-

expression of an OVA transgene resulted in enhanced T cell responses to the antigen and regression of tumours in an OVA-producing tumour mouse model. By contrast, activation of JNK produced minimal up-regulation of co-stimulatory molecules and did not enhance T cell responses. Activation of ERK in DC, using a dominant negative MEK-1, resulted in an increase in TGF-β expression and no increase in co-stimulatory molecule expression. CD8+ T cell responses to co-encoded antigen (OVA) were reduced and a T regulatory population was generated by vaccination which expanded substantially on re-exposure to antigen[335].

The targeted activation of NFκB has been used by other groups to enhance DC activation and T cell responses. Andreakos *et al* overexpressed NFκB inducing kinase (NIK) in DC using adenoviral vector transduction, resulting in increased CD80, CD86, IL-12, IL-15 and IL-18 expression. This enhanced T cell responses to co-encoded GFP, both *in vitro* and *in vivo*[343]. Others have shown that chromosome 1 open reading frame 190 (C1orf190) activates the classical NFκB pathway when overexpressed in monocyte derived human DC by transfection, resulting in secretion of pro-inflammatory cytokines including IL-12p70 and IL-6[344].

An alternative strategy of NFκB activation is to silence its negative regulators leading to sustained activation. Small interfering RNA (siRNA)-mediated silencing of A20, a negative regulator of NFKB, prevents this molecule from deactivating (by ubiquitin editing) components of the TNFR and TLR signalling pathways[345]. Delivery of siRNA using transfection or lentiviral vectors simulated persistent TLR activation of NFκB and results in up-regulation of CD80, CD86, CD40, IL-6 and TNFα[346]. This strategy can be used to generate superior anti-tumour CTL and CD4+ T cell responses. However, A20 silencing also leads to IL-10 up-regulation and potential immunosuppression, and inhibition of both A20 and IL-10 was required to stimulate effective melan-A-specific CD8+ T cell responses in a mouse melanoma model[347].

The NFκB pathway can be manipulated using non-endogenous viral proteins. We and others have previously shown that vFLIP from KSHV is a potent activator of NFκB[348–351]. vFLIP binds to NEMO (IKKγ) inducing a conformational change that leads to IKK activation and NFkB activation via the classical pathway[350,352]. Others have reported that vFLIP over-expression also results in up-regulation of p100/NF-kappa B2

expression resulting in p52 subunit processing and NFκB activation via the alternative pathway[353].

Activation of the NFκB pathway by vFLIP appears to be highly specific. Microarray analysis of both endothelial cells and primary effusion lymphoma cell lines (in which vFLIP is constitutively expressed) reveal a consistent pattern of up-regulation of only those genes with NFκB responsive promoter elements[354,355]. Since activation occurs far downstream in the classical pathway and is independent of NFκB activation by cytokine signalling pathways[356], TRAF6, TAK1 or linear ubiquitination associated complex (LUBAC)[357], constitutive expression of vFLIP will result in sustained and specific NFκB activation without interference from other signals. Some regulatory feedback does occur however from NFκB mediated up-regulation of A20 which in turn inhibits IKK activation[358].

Lentiviral vectors expressing vFLIP activate transduced DC and increase expression of CD80, CD86, CD40, ICAM-1, TNFα and IL-12 secretion[359]. Co-expression of vFLIP and OVA in a lentiviral vaccine resulted in up to 10-fold greater CD8+ T cell responses than observed with LV expressing OVA alone. LV expressing vFLIP-OVA immunised mice showed prolonged survival in a OVA-expressing tumour challenge model and a single immunisation with vFLIP-OVA reduced parasite counts after subsequent challenge with an OVA-expressing leishmaniasis *donovani* strain[124]. Expression of vFLIP in a non-integrating LV vaccine has also been shown to enhance T cell responses against co-encoded antigen[126].

3.2 Aims

The varying effects of p38, JNK, ERK and NFκB activation upon DC activation are summarised in Figure 3-1. Of these, only NFκB activation appears to increase both co-stimulatory molecule expression and secretion of pro-inflammatory cytokines. This pattern of co-stimulation should favour the generation of effective cytotoxic CD8+ T cell responses and TH-1 skewed CD4+ T helper cell responses that are thought to be essential in anti-influenza responses.

In the experiments described in this chapter we aimed to test the efficacy of lentiviral vectors in generating antigen–specific CD8+ and CD4+ T cell responses against encoded Influenza A NP (from A/PR/8/34) as a means of generating cross-strain protective immunity against influenza A in mice. We also wished to determine if the vFLIP would enhance DC activation and T cell responses in mice, and whether this corresponded to enhanced protection against lethal influenza challenge using a model of BALB/c mouse infection with A/PR/8/34.

3.3 Results

3.3.1 Influenza NP and vFLIP are expressed in 293 T cells and BALB/c DC following LV transduction

LV constructs are shown in Figure 3-2A. All mouse vaccination experiments were performed with dual promoter lentiviral constructs using spleen forming virus (SFFV) promoter driving insert 1 and ubiquitin (UBI) promoter driving insert 2.

Figure 3-2B shows that transduction of human 293T cells *in vitro* with varying doses of LV expressing influenza NP gave rise to readily detectable 64 kD protein on western blot two days later. vFLIP expression was also readily detected in 293T cells after transduction. This was also the case in BALB/c bone marrow derived DC confirming adequate functioning of the SFFV and ubiquitin promoters in both cell types.

To demonstrate IKK-dependent NFκB activation by LV expressing vFLIP, WT pre-B cells and IKK knock-out pre-B cells (1.3E2) were transduced with lentiviral vectors expressing vFLIP-GFP or Null-GFP. RelA nuclear localization was measured using a quantitative confocal microscopy assay (see methods page 91). Image analysis was used to separate GFP+ and GFP− cells and measurement of nuclear/cytoplasmic ratio of RelA staining in individual cells was used as an estimate of RelA nuclear translocation[356]. This showed RelA nuclear translocation in vFLIP transduced WT but not IKK KO pre-B cells or pre-B cells transduced with GFP alone (Figure 3-2D).

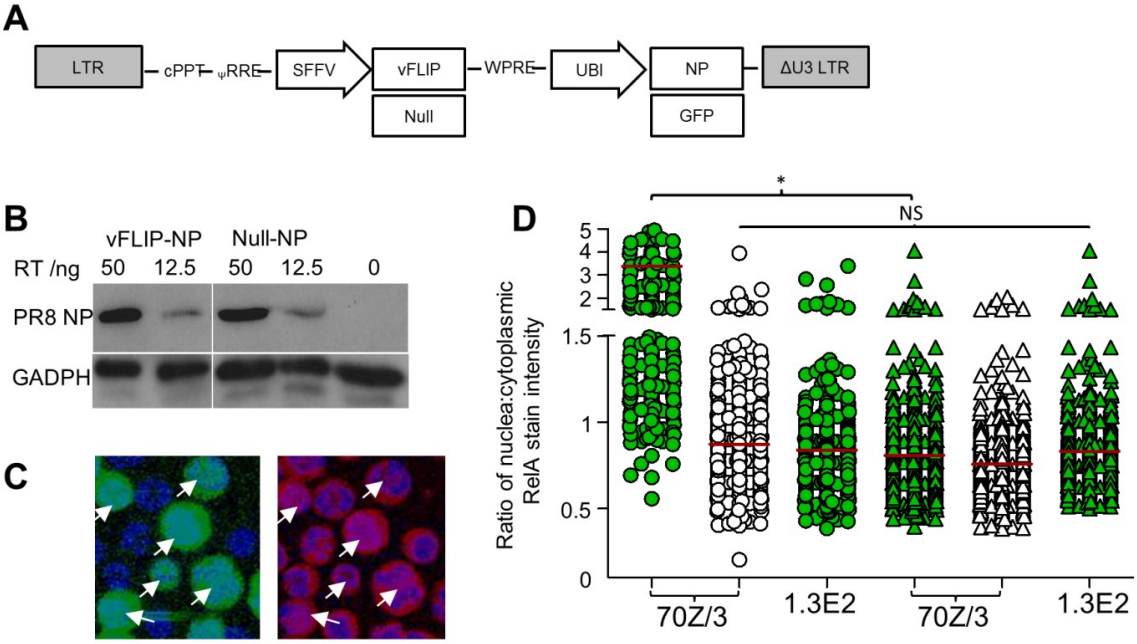


Figure 3-2 (A) Lentiviral vector constructs. **(B)** Western blot showing dose-dependent expression of influenza NP in 293T cells. **(C)** Confocal assay of nuclear RelA location in Pre-B cells transduced with vFLIP-GFP. Arrows indicate GFP+ transduced cells. Left panel shows GFP signal. Right panel shows RelA distribution staining. (**D)** Analysis of RelA staining in nuclear:cytoplasmic region ratios (see methods page 91). 70Z/3 are wild-type pre-B cells. 1.3 E2 cells are IKKγ knock-out pre-B cells. Green shapes indicate GFP-transduced cells. Cells were transduced at an MOI of approximately 0.5 with vFLIP-GFP (circles) or Null-GFP (triangles). Data from one experimental set are shown. Mean values for nuclear:cytoplasmic RelA ratios from three independent repeats were compared with a Students T test (significance level indicated above brackets). A significant increase in nuclear:cytoplasmic RelA ratios was seen in vFLIP-transduced, IKKγ+ cells only.

3.3.2 Co-stimulatory molecule expression in BALB/c DC is increased by vFLIP

We aimed to corroborate the results of Rowe *et al* in BALB/c DC using a panel of six markers of DC activation (CD80, CD86, CD40, ICAM-1, PDL-1, MHC II). A minimum of 6 independent experiments using BM derived DC poled from 2 mice was used on each occasion to reach a quantitative estimate of the fold increase induced in each marker. Transduction of BALB/c DC with LV expressing vFLIP-GFP resulted in significant up-regulation of co-stimulatory molecules, programmed cell death ligand-1 (PDL-1) and MHC II compared with un-transduced DC (Figure 3-3). In nearly all cases (with the exception of PDL-1) up-regulation induced by vFLIP was not significantly different from that induced by LPS. Transduction with LV expressing GFP alone resulted in significantly less co-stimulatory molecule up-regulation.

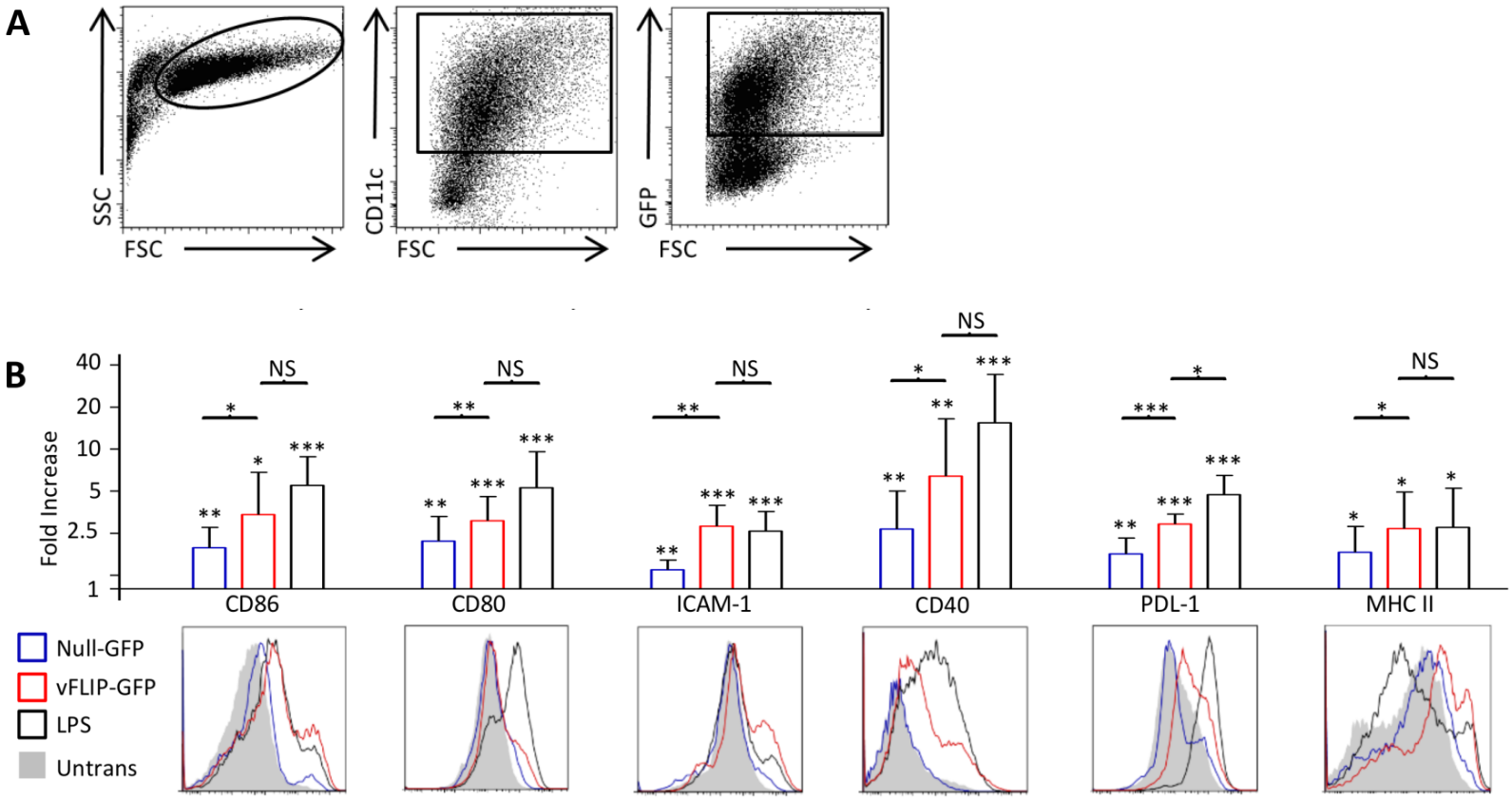


Figure 3-3 (A) Gating strategy of BM-derived DC analysed on day 4 after transduction with vFLIP-GFP or Null-GFP. LPS treated or untransduced DC were gated on CD11c alone. **(B)** Fold increase of expression (note exponential scale) of co-stimulatory (and PDL-1) molecules on DC after transduction or LPS treatment. The fold increase in expression was calculated from the MFI of transduced/treated DC divided by that of untransduced DC in the same experimental set.

Where expression was bimodal (CD86, CD40, ICAM-1, MHC II), % of positive DC were compared with untransduced populations. Significance levels are indicated by stars over where the factor of increase was significantly greater than 1 (i.e. no increase). No significant difference in co-stimulatory molecule expression was observed between LPS matured DC and vFLIP-GFP transduced populations. The data shown is from 6 independent repeats using BM-derived DC pooled from 2 mice on each occasion.

3.3.3 vFLIP enhances functional CD8+ and TH-1 CD4+ T cell responses to influenza NP in BALB/c mice

A dose of 50 ng RT was given for subcutaneous (SC) vaccination following titration experiments which established a fall in CD8+ T cell responses below 25ng RT by ELIspot (data not shown). CD4+ and CD8+ T cell responses to SC vaccination were measured 14 days later by IFNγ ELIspot or intracellular cytokine staining for IFNγ and TNFα following overnight incubation with class II restricted (NP_{57-78}) or class I ($NP_{147-155}$) peptide respectively. Figure 3-4B shows significantly greater numbers of IFNγ+ CD8+ T cells generated by vaccination with vFLIP-NP compared with Null-NP, as measured by both ELISpot and intracellular cytokine staining.

NP-specific CD8+ T cells were quantified by pentamer without overnight re-stimulation. This revealed a trend towards greater numbers of NP-specific CD8+ T cells in the vFLIP-NP versus Null-NP group, but this did not reach significance (Figure 3-4C). No differences were found in IL-7R expression or CD62L (data not shown). Pentamer positive CD8+ T cells were also stained for granzyme B (GzmB) and Ki67 after re-stimulation, which indicated greater on-going proliferation and cytotoxic potential in NP-specific CD8+ T cells 14 days after vaccination (Figure 3-4C).

Homologous dual vaccination 14 days apart resulted in neither increased numbers of pentamer positive CD8+ T cells nor IFNγ positive splenocytes on re-stimulation *in vitro*. However, a greater proportion of effector memory $CD62L^{LO}$ T cells was observed in the spleen 2 weeks after final vaccination with dual vaccination with vFLIP compared with a single dose (Figure 3-5). A significant difference in CD62L expression between dual and single Null-NP vaccinated mice was not observed.

Although numbers of IFNγ+ or TNFα + CD4+ T cells were lower than CD8+ T cells after vaccination, a significant difference was observed between vaccination with vFLIP-NP and Null-NP (Figure 3-6A). A small population of GzmB + CD4+ T cells was identified in mice vaccinated with vFLIP-NP but not Null-NP. To determine the TH-1 versus TH-2 response of mice to vaccination, splenocytes were harvested at day 14 and incubated in media for a further 4 days with the class II restricted NP_{57-78} peptide. Supernatants were then analysed by cytokine bead array. Splenocytes from mice vaccinated with

vFLIP-NP and re-stimulated with NP_{57-78} peptide produced comparable quantities of TNFα and IFNγ to splenocytes from unvaccinated mice stimulated with ConA (Figure 3-6B**).** The IFNγ:IL-4 ratio in vFLIP-NP supernatants was also significantly greater than in Null-NP after vaccination (mean 3.564 versus 1.04, p=0.02) indicating a skewing of the T-helper response towards a TH-1 phenotype with vFLIP. Dual vaccination 14 days apart showed similar results in CD4+ T cell responses 14 days after the last vaccination (not shown).

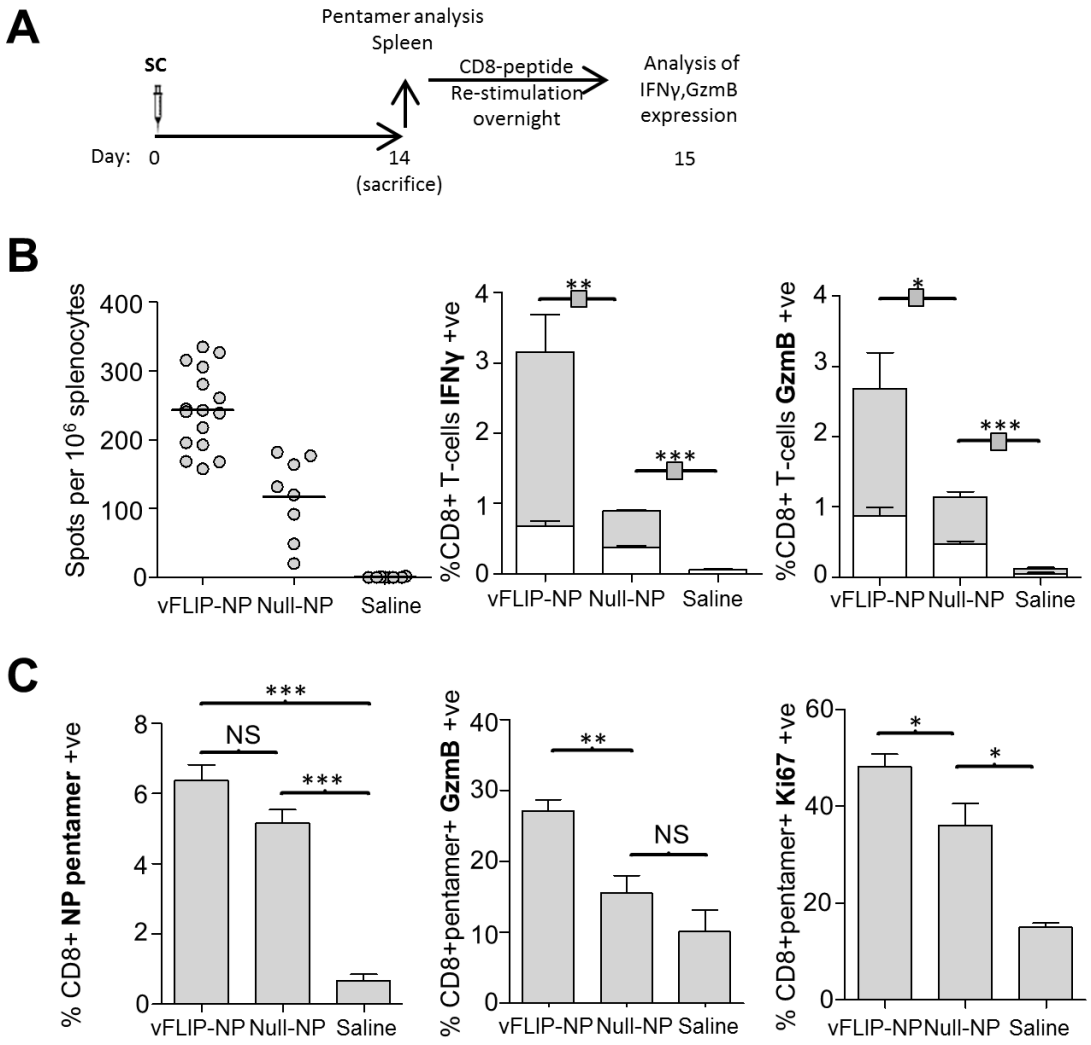


Figure 3-4 CD8+ T cell responses after vaccination with LV according to the schedule shown in **(A)**. **(B)** IFNγ ELISpot and intrancellular staining for IFNγ and GzmB after re-stimulation overnight with $NP_{147\text{-}155\ class\ I}$ restricted peptide. Unstimulated populations are superimposed with clear bars. **(C)** NP pentamer+ CD8+ T cells analysed for GzmB and Ki67 expression.

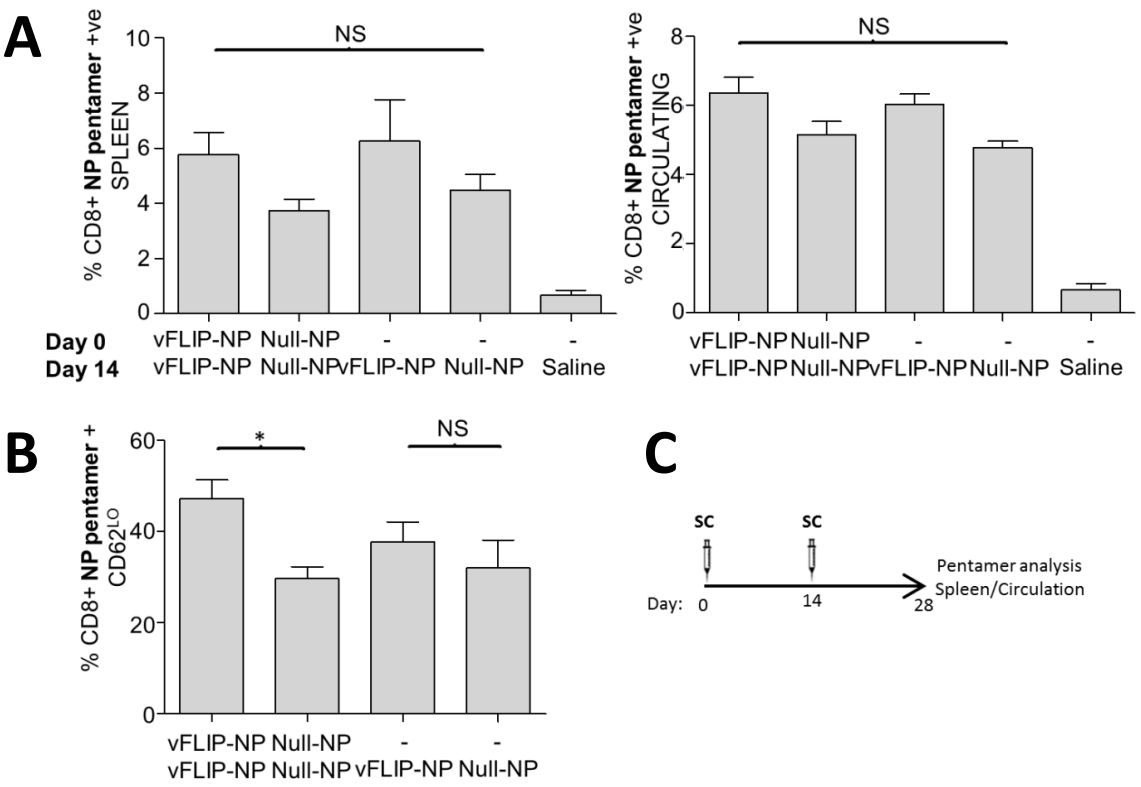


Figure 3-5 (A) $NP_{147-155}$ pentamer percentages of total CD8+ T cells after single and dual vaccination with vFLIP-NP or Null-NP according to the schedule in **(C). (B)** % $CD62L_{LO}$ (effector phenotype) NP pentamer+ CD8+ T cells after single or dual LV vaccination.

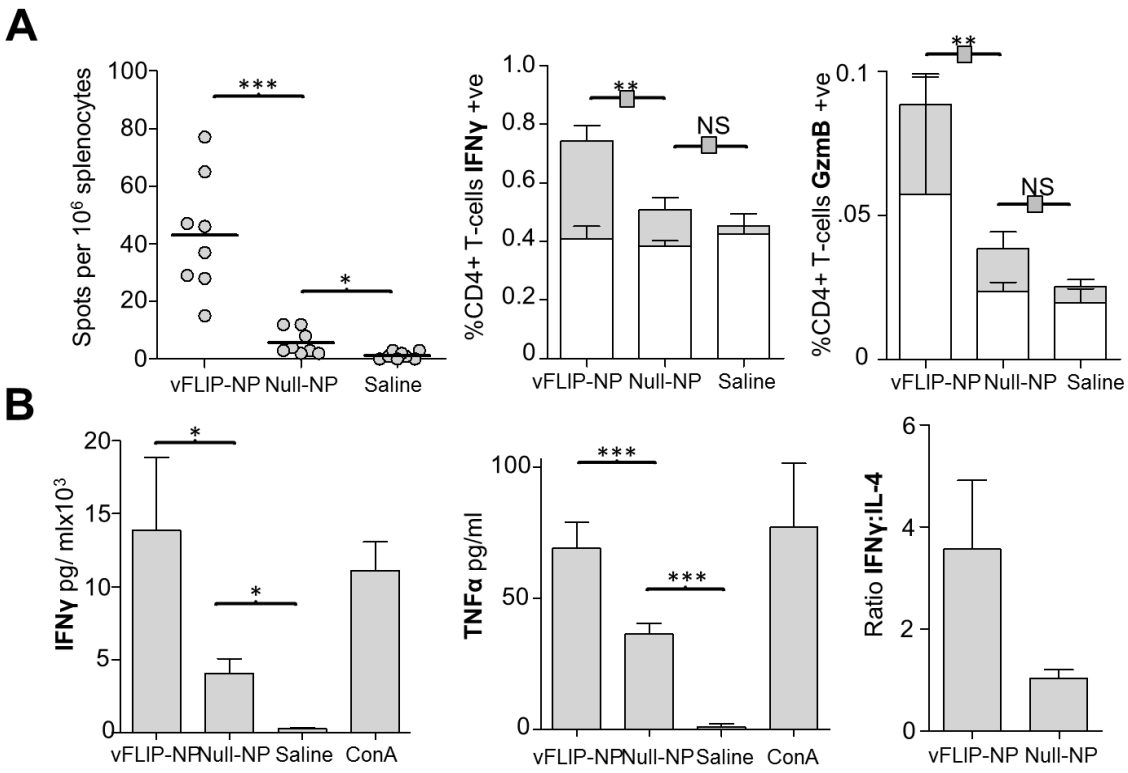


Figure 3-6 CD4+ T cell responses after LV vaccination. **(A)** ELISpot and IFNγ and GzmB expression analysis by FACS after overnight re-stimulation with class II restricted NP peptide. Unstimulated populations are superimposed with clear bars. **(B)** Concentrations of IFNγ and TNFα in supernatant of splenocytes re-stimulated with class II restricted peptide for 4 days as described in the methods.

3.3.4 LV encoding NP confer protection against lethal A/PR/8/34 challenge.

To determine whether the superior CD8+ and CD4+ T cell responses induced by vFLIP-NP versus Null-NP conferred greater protection against live influenza challenge, groups of mice (minimum n=16) were vaccinated with either single subcutaneous injections of 50 ng RT of LV or two vaccinations two weeks apart. Mice were challenged 2 weeks later with an intranasal $2xLD_{50}$ dose of mouse-adapted A/PR/8/34 (corresponding to 2500 PFU) and weights and clinical sign scores recorded from day 3 onwards. Mice losing more than 25% of weight were deemed irrecoverable and euthanized under the terms of our Home Office animal license. Monitoring finished when all surviving mice had regained baseline weight.

Mice vaccinated with vFLIP-NP demonstrated significantly greater survival than mice vaccinated with Null-NP (81.2% vs. 12.5% for dual vaccination regimens, $p<0.001$, 53.8% vs. 11.11% for single vaccination, $p=0.009$, Mantel-Cox test). Dual vaccination with vFLIP-NP appeared to confer superior protection although this did not reach significance. All unvaccinated mice had died or fallen below 75% baseline weight by day 9.

Despite the survival benefit of vFLIP-NP vaccination weight loss was still considerable, with a mean maximum weight loss of 17% at day 6. Mice also developed clear signs of infection including tachypnoea, piloerection and hunched posture. Clinical signs of infection resolved rapidly after day 6, with mice also recovering weight at around 1 g per day.

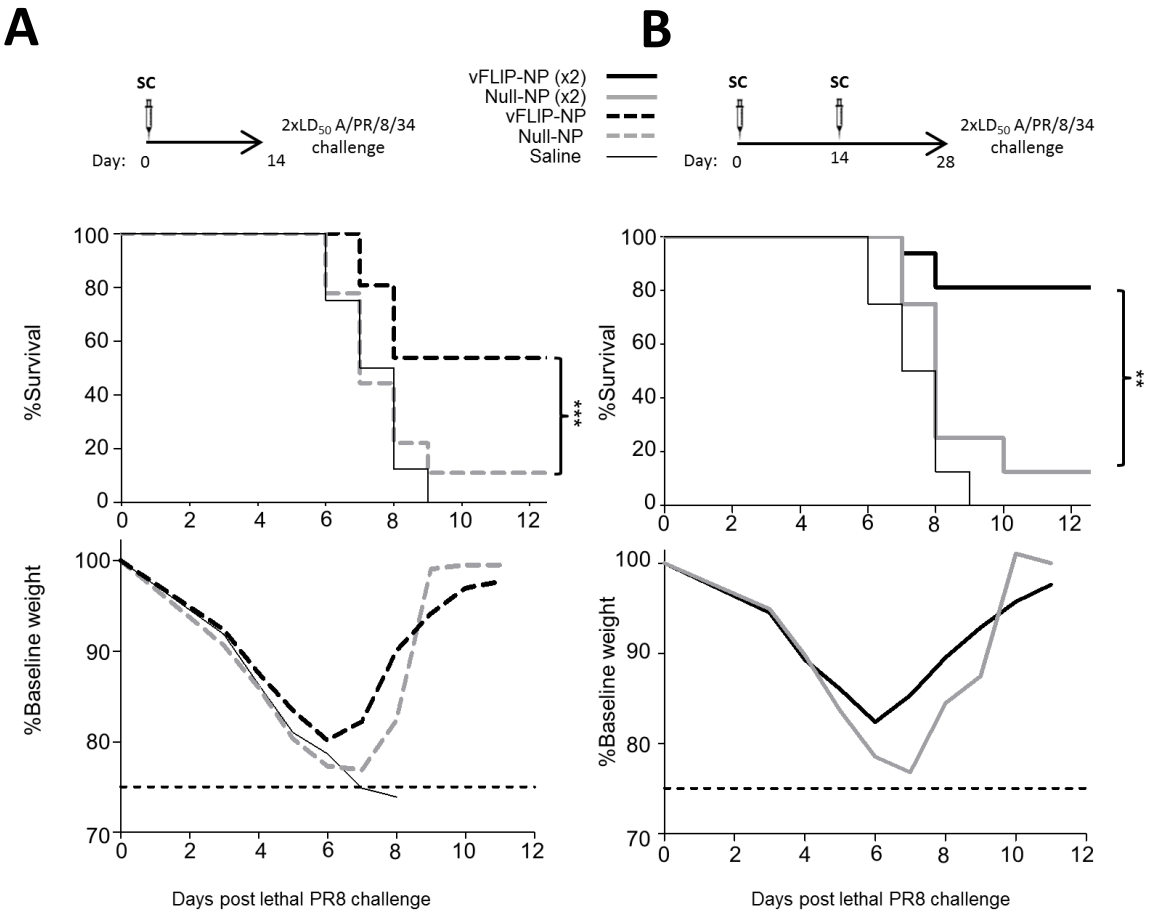


Figure 3-7 Survival and weight loss in surviving mice following single **(A)** or dual **(B)** vaccination with vFLIP-NP or Null-NP. All mice were challenged with a lethal dose of A/PR/8/34 and weights monitored from day 3. Mice losing more than 25% of baseline rate were deemed irrecoverable and sacrificed. Both Mantel-Cox tests and Gehan-Breslow-Wilcoxon Test produced similar p-values for observed differences in survival.

3.4 Summary

LV expressing vFLIP activate NFκB in an IKK-dependent manner and activate DC to an equivalent degree as LPS. LV expressing NP are capable of generating CD8+ and CD4+ T cells against the encoded antigen after SC administration. Numbers of antigen-specific CD8+ T cells were not increased by co-expression of vFLIP, but numbers of functional CD8+ T cells, as measured by IFNγ secretion or GzmB expression after re-stimulation were significantly increased. vFLIP also skewed the CD4+ T cell response towards a TH-1 phenotype. These differences corresponded to greater protection against lethal challenge with A/PR/8/34 in vFLIP-NP versus Null-NP vaccinated mice. Vaccinated and unvaccinated mice were clinically indistinguishable until day 5-6 when vaccinated survivors began to rapidly recover weight.

3.5 Discussion

LV have been previously shown to induce cellular immunity against numerous antigen targets (Table 1-3, page 51). LV vaccines shave been effective in generating antibody responses against West Nile Virus envelope protein[130] and HIV gp120[108] and cellular responses against HIV[360], SIV[361] GAG and a leishmaniasis strain expressing OVA[359]. One study has demonstrated reduction in SIV titres following challenge of macaques with SIV, following vaccination with LV expressing SIV GAG, when used in a homologous prime-boost regimen[362].

Prior to this study, however, the ability of an LV vaccine to generate protective T cell responses against an acute viral infection had not been tested. A/PR/8/34 is a mouse-adapted influenza strain with high pathogenicity which provides a robust test of T cell mediated protection. vFLIP-NP vaccination compares favourably with previously described means of generating T cell mediated protection against this strain of influenza in mice as summarised in Table 3-1 below. Notwithstanding the factors described in the introduction that make inter-experimental comparisons difficult in this field, an analysis restricted to studies using NP as a target antigen, BALB/c mice

and a A/PR/8/34 challenge reveals that the degree of survival correlates negatively with challenge dose by LD_{50} (Figure 3-8). It is unclear whether this is a linear or negative exponential correlation, but all experiments using a challenge greater than 5x LD_{50} reported very low or no survival. This underlies an important limitation in protection mediated by systemic T cell vaccines, in that it can be readily overwhelmed by high challenge doses. Another consistent finding is despite the highly variable degrees of weight loss observed in these studies, the time point at which the nadir of weight is reached is remarkably constant at 6.7 ± 0.8 days (μ ± 95% CI). Therefore, *irrespective of the size of the T cell response generated by vaccination,* there appears to be at least a 5-day window after infection wherein systemic T cell immunity has little bearing on the development of clinical disease.

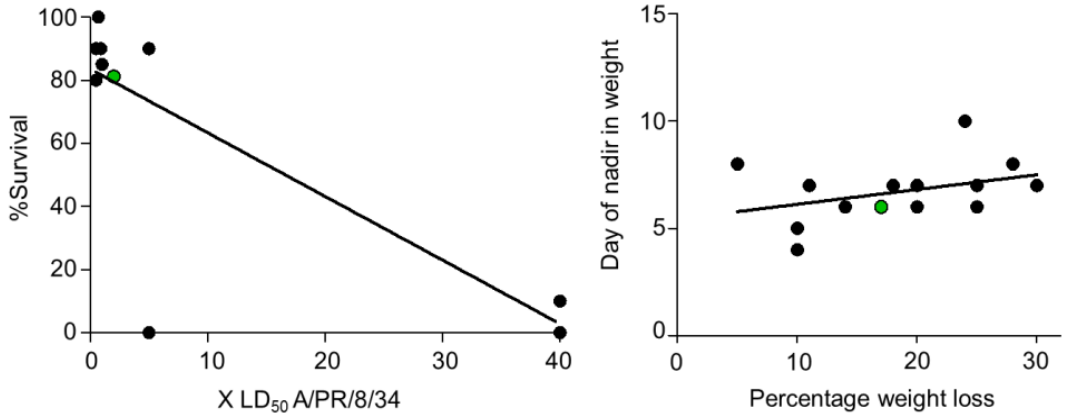


Figure 3-8 Summary of survival rates and timing of nadir of weight loss in vaccination experiments described in Table 3-1 that use influenza NP as a target antigen and A/PR/8/34 challenge. The green circle indicates survival and timing of maximal weight loss after vFLIP-NP SC vaccination.

Table 3-1. Summary of mouse vaccination experiments using DNA or viral vectors expressing influenza NP

Reference	Antigen	Vaccine modality	Mouse species	Challenge virus/ Dose	Survival	Minimum weight loss	Nadir of weight loss (day)	Main findings
Fu *et al* 1997[363]	NP NP_{mut}	DNA	BALB/c	A/HK/68 (H3N2)/10xLD_{50}	100%	Not measured	N/A	DNA immunisation with mutated dominant NP epitopes still capable of inducing protective CD8 immunity.
Fu *et al* 1999[364]	NP	DNA (varying doses)	BALB/c	H3N2 A/HK/68 1xLD_{70} H1N1 A/PR/8/34/ 1 xLD_{70}	100%	20%	7	Protection and T cell response is dose-dependent
Ulmer *et al* 1998[365]	NP	DNA	BALB/c	H3N2 (A/HK/68) 1xLD_{50}	100%	20%	6	Protection reduced to 50% with CD8+ T cell depletion
Robinson *et al* 1997[366]	NP	DNA	BALB/c	H1N1 PR/8/34 10xLD_{50}	0%	Not measured	N/A	High challenge dose of high pathogenicity virus results in no detectable protection
Chen *et al* 1999[367]	NP	DNA	BALB/c B10 C3H	H1N1 PR/8/34 40xLD_{50}	BALB/c 10% B10 0% C3h 15%	26.7 (day 7) 26.4(day 7) 25.2(day 7)	N/A	NP-DNA fails to confer protection in context of a high challenge dose of mouse-adapted influenza

Chen *et al* 1998[368]	NP	DNA	BALB/c	H1N1 PR/8/34 40xLD_{50}	0%	N/A	N/A	NP-DNA fails to confer protection in context of a high challenge dose of mouse-adapted influenza
Saha *et al* 2006[369]	NP fused with HSV VP22	DNA	BALB/c	A/Udron/72 5xLD_{50}	NP 20% NP-VP22 80%	25% 11%	6 7	Tegument protein VP22 enhances T cell response against NP and protection. CD8+ depletion impairs protection.
Roy *et al* 2007[370]	NP	AdC7 (chimp adenovector) AdHu5 (human adenovector)	BALB/c	H5N1(Vietnam/1203/04 or Hong Kong/483/97) 100x LD_{50} H1N1 PR/8/3410xLD_{50}	PR8 90-100% Viet04 50-80% HK97 20-30%	Not assessed	N/A	Chimp and human adenoviral vectors expressing NP give comparable protection against lethal challenge
Tsuji *et al* 1998[371]	NP epitopes	Recombinant Sindbis virus	BALB/c	H1N1 A/WJN/33	Not assessed	Not assessed	N/A	Recombinant Sindbis virus generates CD8+ T cell responses against incorporated NP epitopes
Laddy *et al* 2008[372]	NP	DNA with electoporation		H1N1 PR/8/34 10xLD_{50}	H1N1 -90%	25%	7	Also demonstrated protection in ferrets
Bender *et al* 1998[373]	NP	DNA	BALB/c	H3N2 A/HK/68 10^3 $TCID_{50}$	67%	24%	10	Similar CTL responses in young and aged mice but significantly less protection in the latter, attributed to non-CTL factors.

Ulmer *et al* 1994[374]	NP	DNA	BALB/c	"lethal doses"	80%	Not assessed	N/A	Demonstrates dose dependent CTL responses and protection.
Epstein *et al* 2002[375]	NP and M1	DNA	BALB/c	H5N1 1997 HK/156 HK/483 HK/485 and HK/486 10-100x MID_{50}	100% to 100 MID_{50} of intermediate virulence HK/156 0% to 100 MID_{50} of high virulence HK/483 50% to 10xMID_{50} HK/483	14.2% HK/156	6	Demonstrates the sensitivity of T cell mediated protection to the dose and virulence of the challenge strain.
Bot *et al* 1998[376]	NP	DNA	BALB/c	H1N1 PR/8/34 1xLD_{100} H3N2 A/HK/68 $1.5x10^5$ $TCID_{50}$	Adults: 85% PR8, 10% HK68 Neonates: 30% PR8, 0% HK68	Not assessed	N/A	T cell responses and protection attenuated in newborn mice compared with adults

Epstein *et al* 2005 [377]	NP	DNA prime +/- Adenovector boost	BALB/c	H5N1 HK/483/97 $10xMID_{50}$ H1N1 PR/8/34 $200xLD_{50}$	NP DNA 100% against HK483 0% against PR8 NP DNA + NP Ad5 70% against PR8	NP DNA:25% NP DNA/Ad5 :10%	NP DNA: 7 NP DNA/Ad5: 4	DNA Prime Ad5 boost induces greater CD8 T cell responses than DNA alone, increases survival, shortens disease and lessens severity.
Altstein *et al* 2006[378]	NP	Vaccinia virus	BALB/c	H3N2/$5xLD_{50}$ or $1XLD_{50}$	100% 1 x LD_{50} 80% $5xLD_{50}$	Not assessed	N/A	A co-encoded rapid proteolysis signal increases MHC expression and improved T cell responses
Moraes *et al* 2011[379]	NP	Adv5 +/- 4-1BBL	C57BL/6	H1N1 PR/8/34 $1xLD_{50}$	Adv5 4-1BBL NP: 80% Adv5 NP: 50%	Adv5 4-1BBL NP 18% Adv5 NP: 28%	Adv5 4-1BBL NP :7 Adv5 NP: 8	4-1BBL co-expressed with NP in an adenovector enhances T cell responses, protection and their longevity compared with NP alone
MacLeod *et al* 2011[380]	NP	Protein +/- Alum and/or monophosphoryl lipid A	BALB/c	H1N1 PR/8/34 150 PFU	Sub-lethal challenge in controls	5% with Alum + MPL 15% with NP alone	8	Commonly used adjuvants can enhance T cell responses to NP recombinant protein for improved protection against sub-lethal challenge

Jimenex *et al* 2007[381]	NP	Liposomal DNA delivery	BALB/c	H1N1 PR/8/34 $1xLD_{90}$	NP alone 20% protection	30%	7	Protection improved with inclusion of M2
James *et al* 2007[382]	NP and/or M1/M2	DNA +/- DNA encoding α/β interferons	BALB/c	H1N1 PR/8/34 $10^{1.5}TCID_{50}$	Sub-lethal challenge in controls	10%	5	Co-delivery of DNA encoding α/β interferons reduced viral titres and weight loss
Degano *et al* 1999[383]	NP	DNA +/- MVA	BALB/c	H1N1 PR/8/34 100 HA units (approx. $1xLD_{50}$)	DNA-NP+MVA-NP: 90-100% MVA-N: 0%	9-12%	N/A	MVA-NP alone did not protect. MVA-NP boosted DNA-NP superior to DNA-NP alone (similar survival but less weight loss)

This is most likely a consequence of the location of memory T cells rather than their quantity or functionality. Both effector memory T cells and central memory T cells contribute to the secondary response to influenza but do so in temporally distinct phases (as discussed in the introduction to the thesis) with lung-resident and recruited effector memory T cells (T_{EM}) forming an early functional but non-proliferative response, followed by a rapid expansion of antigen specific T cells between days 4 and 7 resulting from recruitment of T_{EM} that have differentiated from proliferating central memory T cells (T_{CM}) in secondary lymphoid tissues. This latter phase occurs sooner in the secondary response due to the more rapid dynamics of T_{CM} proliferation in response to antigen presentation in lymphoid tissue compared with the primary response. Woodland *et al* showed that If secondary T cell responses to influenza in mice are tested by heterosubtypic challenge 8 months after the primary infection, then substantial T cell recruitment to the airway does not occur until between day 5 and 7 post-infection (Figure 3-9) (versus day 7-10 in the primary response)[384]. Since tissue resident antigen-specific T cells have waned 8 months after primary infection, this scenario is analogous to T cell immunity generated by systemic vaccination (intramuscular, subcutaneous or peritoneal), following which antigen–specific mucosal T cell populations are known to be no more enriched than in the general circulation[385]. The substantial recruitment of secondary effector T cells to the airway between day 5-7 corresponds to the consistent time-point at which recovery is observed in mice systemically vaccinated against NP.

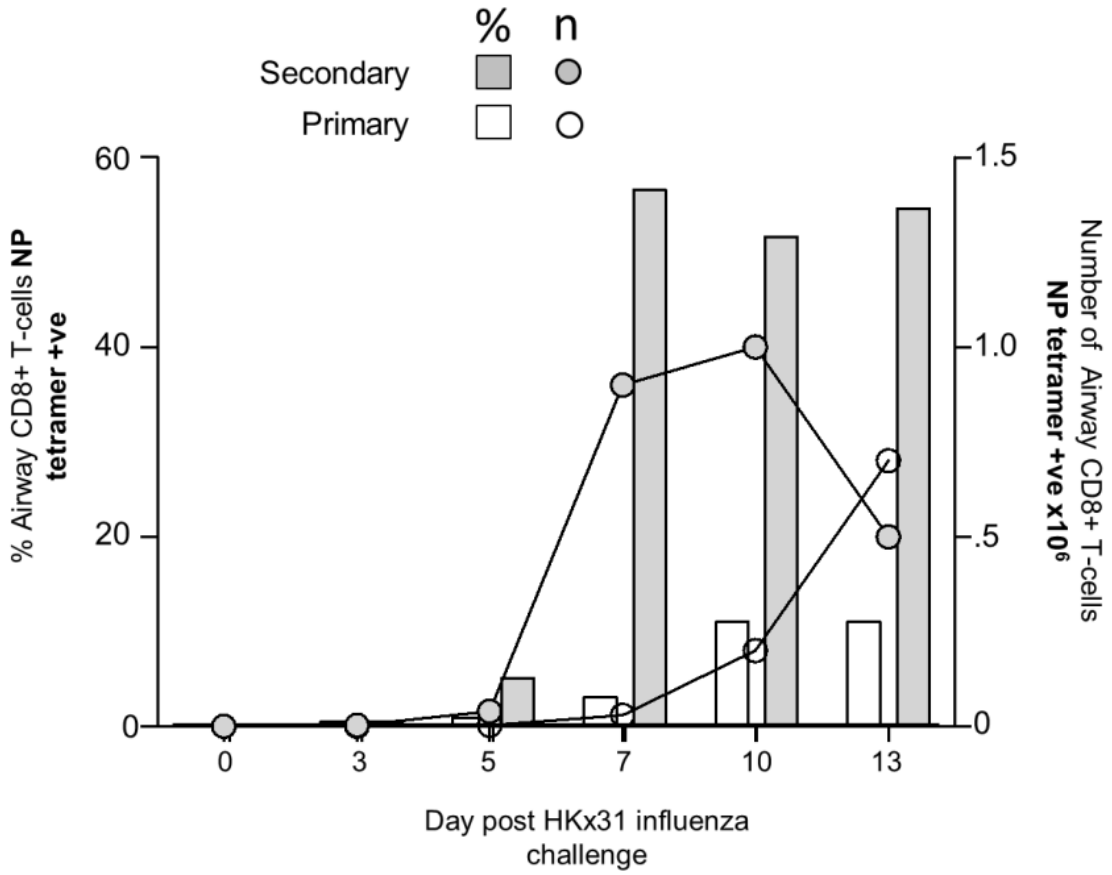


Figure 3-9 Primary and secondary T cell responses to heterosubtypic strains in the airway. Adapted from data in Woodland *et al*[384].

It may therefore be inferred that protection is determined not only by the quantity and functionality of the T cell response, but also by its timing and anatomy. Increasing the numbers of circulating T cells after vaccination may be one means of improving protection. Homologous prime-boost vaccination with LV has been shown to increase T_{EM} cell responses against encoded OVA[386], Melan-A[387], HIV-1 poly-epitope[388] and HIV-1 gag[389], exploiting the low anti-vector antibody responses induced by VSV-G pseudotyped LV which permit effective transduction with subsequent doses[362]. We found in this study that numbers of NP-specific CD8+ T cells were not increased by dual compared with single vaccination. However, we did observe an increase in the proportion of $CD62^{LO}$ effector phenotype NP-specific T cells with dual vaccination. Given that effector T cells are recruited to the lung and airway during influenza infection during the first few days of infection, this may explain the trend towards superior survival seen with dual versus single vFLIP-NP vaccination.

In the context of anti-tumour immunity or protection against slowly replicating infections, timing and location of memory T cells may be less crucial than in acute viral infection. Nevertheless, development of T cell vaccines for HIV, which replicates substantially slower than influenza, has pointed towards the benefits of priming T_{EM} that will persist at mucosal sites of infection[390]. In influenza, an effector T cell population at the site of infection prior to challenge may have a dramatic impact on viral propagation, since the impact of the killing rate of infected cells on the production of infectious virions is inversely proportional to the number of infected cells (which is at its lowest after the first round of infection). However, the factors that determine the development and persistence of tissue-resident T_{EM} cell population in the lung and airway are incompletely understood. The observation that these are established by pulmonary infection with influenza but not infection by other routes (such as intravenous or intraperitoneal) would imply that mucosal vaccination would be the best means of establishing mucosal T cell populations[391]. In terms of T cell function, the development of mucosal T cell vaccines for TB, RSV (lung) and HIV (gastrointestinal and vaginal) have been based on the empirical assumption that mucosal priming will generate T cell memory cells functional adapted to combat mucosal infection.

In the following chapter the potential of LV to induce mucosal T cell populations for protection against influenza is investigated.

4 Mucosal vaccination with lentiviral vectors

4.1 Introduction

4.1.1 Immune homeostasis in the lung

In addition to distinguishing self from non-self, a fundamental role of the immune system is to distinguish potentially harmful from non-harmful antigen. This is particularly challenging at the mucosal surfaces where exposure to foreign antigen is varied and continuous. The lungs are the largest mucosal surface area of the body and disruption of its delicate architecture by inflammation has a high cost in impaired gas exchange.

Minimising the antigen exposure levels to near-sterility (compared with the gastrointestinal mucosa, for example) partially addresses this problem and is achieved through a number of adaptations of the mucosal barrier, including the mucociliary clearance system and the secretion of soluble agents with anti-bacterial and anti-viral activity such as lysozyme, surfactant, α and β defensins, collectins and lactoferrin. This reduces the antigen load sampled by DC, which are absent from the airway lumen but can access surface antigen in small airways through transepithelial dendritic processes[392]. Alveolar macrophages, by contrast, constitute 95% of the cellular component of broncho-alveolar lavage (BAL) and occupy the mucinous layers of the airway lumen where they have much greater exposure to antigen. They are phagocytically active but markedly limited in their ability to stimulate primary T cell responses. This is due to a number of mechanisms including impaired expression of co-stimulatory molecules[393,394], reduced binding of antigen-specific T cells to AM presenting cognate antigen[395] and active suppression of T cell responses through secretion of IL-10, nitric oxide and TGF-β under steady state conditions[396,397]. The latter may also supress the activation of local DC[398], indirectly and directly limiting both the T cell and also B cells response.

The powerful suppressive effect of AM on pulmonary T cell responses is clearly demonstrated by their depletion. The cytotoxic drug clodronate is avidly taken up by AM when administered intranasally in liposomal form, resulting in their depletion within 48hrs which is sustained for up to one week, with minimal change in other cell populations[399]. Intra-tracheal administration of antigen induced virtually no T cell response in normal animals, but in AM-depleted mice a dramatic increase in T cell proliferative responses was observed[400]. Depletion of AM also enhanced DC activation and the ability to stimulate antigen-specific T cell responses[398].

AM are themselves regulated by inhibitory signals within the alveolar microenvironment. Surfactant protein A (SP-A) inhibits TLR2 and TLR4 signaling by AM[401], blocks TLR-4 activation by LPS[402] and inhibits AM phagocytic activity by activating the inhibitory receptor SIRPα[403]. TLR signalling pathways in AM are also inhibited downstream of the TAK1-TRAF6 complex by mucin-like glycoprotein 1 expression on epithelial cells by an as yet unknown mechanism[404]. Epithelial cells also express CD200, the receptor for which is highly expressed on AM and mediates negative inhibition of the p38 MAPK pathway[405]. AM are also susceptible to immunoregulatory signals from TGF-β and IL-10 secreted by epithelial cells[406,407].

The threshold for activation of innate adaptive immune responses in the lung is thus very high. Cytopathic infection of epithelial cells is likely to be key in overcoming immunotolerance, since this not only initiates pro-inflammatory cytokine release that enhance TLR expression signalling on AM , but also the loss of epithelial barrier integrity exposes less regulated tissue-resident macrophages and DC to antigen.

4.1.2 Vaccine vectors for mucosal T cell immunity

Unsurprisingly, it follows that the vaccines vectors most efficient at generating primary T cell responses in the lung are those derived from pathogens for which this is a natural route of infection.

Live attenuated Influenza strains would seem the obvious choice for the task of generating mucosal T cell responses against influenza. Intranasal LAIV are licensed for

clinical use in children and as discussed above confer protection predominantly through antibody response to HA and NA epitopes matched to circulating seasonal strains. However, they have been consistently shown to induce superior systemic T cell responses against internal virion components to that with trivalent inactivated vaccines in humans[408–411]. It is unknown whether LAIV infection generates directly protective mucosal influenza-specific CD8+ T cell populations, or whether CD4+ T cell help contributes significantly to the strain specific antibody-mediated protection conferred by LAIV. It is a reasonable assumption, however, that mucosal T cell responses to LAIV will be no greater or more lasting than those to influenza itself, and therefore similarly limited in the degree of heterosubtypic immunity they confer. Other viral vectors adapted for mucosal infection and which can be readily adapted to incorporate immunogenic signals may therefore show more promise in generating sustained mucosal T cell populations.

Adenoviral vectors have a natural tropism for the respiratory tract and can cause upper and lower tract infection, typically in childhood[412]. Use of early generation adenoviral vectors for gene transfer to lung epithelium for the treatment of cystic fibrosis revealed strong cellular responses which hampered the longevity of transgene expression[413414]. This ability was later exploited in a mucosal adenoviral vaccine encoding the mycobacterium tuberculosis antigen Ag85 in order to generate lung-based CD4+ and CD8+ T cell responses against TB, which reduced replication upon intranasal challenge[415]. Adenoviral vectors expressing RSV antigens provide an effective intranasal boost of cellular immunity primed by a recombinant salmonella typhimurium, conferring partial protection against live RSV challenge in vaccinated mice. More recently, intranasal vaccination of mice with adenoviral vectors expressing influenza NP generated up to 12.5% of NP-specific CD8+ T cells in the lung. This conferred 80% protection against lethal challenge, but survivors lost approximately 25% of their bodyweight (by day 8) suggesting the pattern of clinical disease was not significantly different from that observed with systemic immunity. Incorporating the external virion target M2 generated an antibody response which enhanced protection to 100% and reduced weight loss in survivors to 10%[416]. The authors speculate that protection is predominantly mediated by CD8+ T cells rather than antibodies against the M2 component since the Ad NP-M2 vaccine is only marginally less efficacious in

IgA-deficient mice. However, their finding of high titres of anti-M2 IgG and the now well-established concept that IgG but not IgA is essential for influenza neutralization[417] brings this conclusion into question.

Pox viral vectors, such as the attenuated Vaccinia strains that were used to eradicate smallpox, were shown to induce mucosal T cell responses after intranasal delivery over 30 years ago[418]. MVA has since been used in numerous studies to generate mucosal T cell responses via gastrointestinal, vaginal and respiratory routes[419]. Intranasal MVA encoding HIV or SIV antigen have been shown to induce greater rectal and vaginal mucosal antigen-specific CD8+ T memory cell infiltration than when administered by IM or SC routes, suggesting a common mucosal immune system wherein T cells primed at one mucosal site will migrate favourably to all mucosal surfaces[420,421].

VSV vectors also have a natural tropism for infection via respiratory routes. Indeed, their ability to transgress the nasal mucosa has led to safety concerns that replicative vectors may infect neuronal tissue via the olfactory bulb[422]. Recombinant attenuated strains of VSV administered intranasally have been used to successfully generate cellular responses against HIV, SIV and CMV antigens detectable in the lung and other mucosal sites[423,424]. Attenuated recombinant VSV can raise effective mucosal antibody responses against HA when delivered intranasally in mice, and these provide robust protection against challenge with influenza strains expressing homologous HA[425]. However, while recombinant VSV expressing influenza NP was able to induce systemic NP-specific CD8+ T cell response when delivered intranasally, it did not induce mucosal NP-specific T cell populations, nor confer significant protection against lethal challenge[426].

Nanoparticle antigen delivery systems have shown considerable promise as mucosal vaccines. A diverse range of lipid based and polymeric nanocarriers have been shown to generate effective humoral and cellular responses to encapsulated antigen when delivered intransally[427]. These either have repetitive intrinsic structures providing carrier-mediated PRR stimulation of antigen presenting cells, or they provide a framework which presents associated antigen in a repetitive pattern to enhance PRR stimulation. Nanocarriers provide resistance to mucosal enzyme antigen degradation and their particulate formulation enhances APC uptake and cross presentation of

encapsidated antigen[428]. Intranasal liposomal formulations have successfully generated cellular responses against plague[429] and anthrax[430] antigens and liposomal encapsidation of DNA expressing TB antigens permits effective generation of TB specific cellular responses in the lung mucosal after intranasal administration, [431]. Nambrini *et al* demonstrated that a fully degradable polypropylene sulphide (PPS) can be coupled to antigen with disulphide linkages which dissociate in a reductive environment, resulting in endosomal release of antigen and enhanced cross presentation[432]. This formulation coupled with OVA and CpG induced very high percentages (44%) of OVA-specific CD8+ T cells in the lungs of intranasally vaccinated mice. This protected mice against lethal challenge with an OVA-expressing recombinant A/PR/8/34, with mice losing an average of just 5% of baseline weight by day 7. Immune Targeting Systems (ITS) have developed a nanoparticle formulation of dilauroylphosphatidylcholine (DLPC) liposomes containing the adjuvants monophosphoryl lipid A (MPL) and trehalose 6,6' dimycolate, encapsulating 6 conserved segments of the PA, PB1,PB2, NP and M1 proteins. Administered intranasally, this induces lung-based responses of around 17% antigen-specific CD8+ T cells which conferred protection against influenza without weight loss. However, the challenge strain used was a non-mouse adapted A/HK/8/68 (considerably less pathogenic in mice than A/PR/8/34) and was given at a dose that failed to kill all unvaccinated mice. In this context, even the empty nanoparticle formulation without peptide was able to induce transient but robust protection, possibly by the mechanism of "innate imprinting" described above. Splenic T cell memory was not generated by intranasal vaccination and survival rates fell to 0% if mice were challenged 8 weeks after final vaccination, questioning the longevity of protection induced by this approach.

Lentiviral vectors have been investigated for their ability to generate mucosal T cell responses by intranasal administration in order to generate HIV-specific T cells in rectal and vaginal mucosae. Intranasal administration of a chimeric simian-human immunodeficiency virus (attenuated by nef deletion) to macaques resulted in larger SHIV-specific CD8+ and CD4+ T cell populations in the rectal and vaginal mucosa than intravenous administration. This completely protected macaques from rectal SHIV challenge, but T cell responses at the respiratory mucosa were not examined[433].

Hashimoto *et al* examined the potential for lentiviral vectors to generate respiratory mucosal T cells specific to the TB antigen MPT51 after intratracheal administration. This resulted in MPT51-specific CD8+ T cells in the lung at frequencies of only 2%, which conferred partial inhibition of growth of TB upon challenge but not protection. Perhaps because of this disappointing result, no other studies have since assessed the potential of intranasal LV for protection against respiratory pathogens.

There has nevertheless been considerable interest in the potential of LV delivered intranasally for gene therapy in cystic fibrosis. Promising early results were achieved with 1st generation VSV-G pseudotyped LV encoding CFTR, which transduced respiratory epithelia *in vitro* albeit with poor efficiency[434]. Pseudotyping of LV with a filoviral envelope (Ebo-Z), however, achieved over 80% transduction of airway epithelium after intratracheal administration to immunocompetent mice[435]. This approach has been used to establish sustained expression of CFTR with functional reversal of the chloride ion transport deficit[436]. Some investigators have reported gene expression after intranasal LV delivery that persists for the lifetime of the mouse[437]. Sustained expression may be in part due to transduction of stem-cell like progenitors, but is also permitted by the consistent lack of T cell responses to the cystic fibrosis transmembrane conductance regulator (CFTR) transgene or other vector components[438].

The observed immunotolerance to intranasally delivered LV transgenes contrasts markedly with the T cell responses observed after subcutaneous administration. It is also surprising given the efficient transduction of alveolar macrophages (AM) by LV observed in several of these studies. For example, Buckley *et al* examined the persistence of GFP expression in the airway epithelia of mice given intranasal LV as newborns or adults[322]. They reported transduction of only 1% of adult respiratory epithelia, but markedly high rates of transduction of AM, approaching 50%. Despite transduction of these APCs, persistent gene expression was observed at 390 days after administration. This tropism of lentiviral vectors for alveolar macrophages was later exploited by Malur *et al* for restoration of surfactant catabolism by AM in an alveolar proteinosis model. This group achieved 79% transduction of AM with a single intratracheal administration of LV[439].

The tolerogenic responses to intrapulmonary administration of LV for gene therapy would suggest this is not an optimal vector for generating strong mucosal T cell responses for protection against influenza. Whilst this may be due to the highly efficient transduction of AM which have a central role in regulation of the adaptive immune response in the lung, this tropism may also present an opportunity for subversion of AM immunoregulation.

4.2 Aims

In the experiments described in this chapter we aimed to test the efficacy of lentiviral vectors in generating NP–specific CD8+ and CD4+ T cell responses in the lung and airway following intranasal administration. We also wished to determine whether NFκB activation, known to be an important pathway in activation of AM as well as DC, may enhance mucosal T cell responses compared with LV expressing NP alone.

4.3 Results

4.3.1 Intranasal LV fails to prime mucosal or systemic T cell responses against NP

Intranasal administration of vFLIP-NP or Null-NP LV (200 ng RT) failed to induce discernible antigen-specific response in CD8+ T cells isolated from lung homogenate 2 weeks later. Indeed, more antigen-specific CD8+ T cells were detected in lung in mice receiving a single VFLIP-NP subcutaneous vaccination than mice receiving a single VFLIP-NP intranasal vaccination (mean 4.157 vs. 0.92, P=0.0002). Splenic or circulating antigen-specific T cells were also not significantly greater than background after intranasal administration of LV, suggesting a failure of both mucosal and systemic T cell priming by the intranasal route (Figure 4-1).

4.3.2 Intranasal LV recalls substantial NP-specific populations to the lung in mice that have previously been vaccinated subcutaneously

The lack of priming observed with intranasal LV contrasted markedly to the T cell response in the lung induced by sequential subcutaneous priming and intranasal boosting (SC-IN). Following SC-IN vaccination with vFLIP-NP more than a third of CD8+ T cells in the lungs harvested 14 days after the last vaccination were NP-specific. Intranasal Null-NP after Null-NP subcutaneous vaccination generated significantly lower proportions of NP-specific CD8+ T cells, although levels were still considerably higher than seen after subcutaneous vFLIP-NP alone. Figure 4-2C compares the relative proportions of NP-specific CD8+ T cells isolated from homogenised lung 14 days after subcutaneous, intranasal or SC-IN vaccination. Whereas all mice were killed by lethal A/PR/8/34 challenge following intranasal vaccination with vFLIP-NP or Null-NP, 100% of mice survived when challenged 14 days after vFLIP-NP SC-IN vaccination. Strikingly, mice developed no outward sign of clinical disease and lost no weight. SC-IN Null-NP vaccination also conferred improved protection compared with SC vaccination with either vFLIP-NP or Null-NP, albeit with evident clinical disease and weight loss.

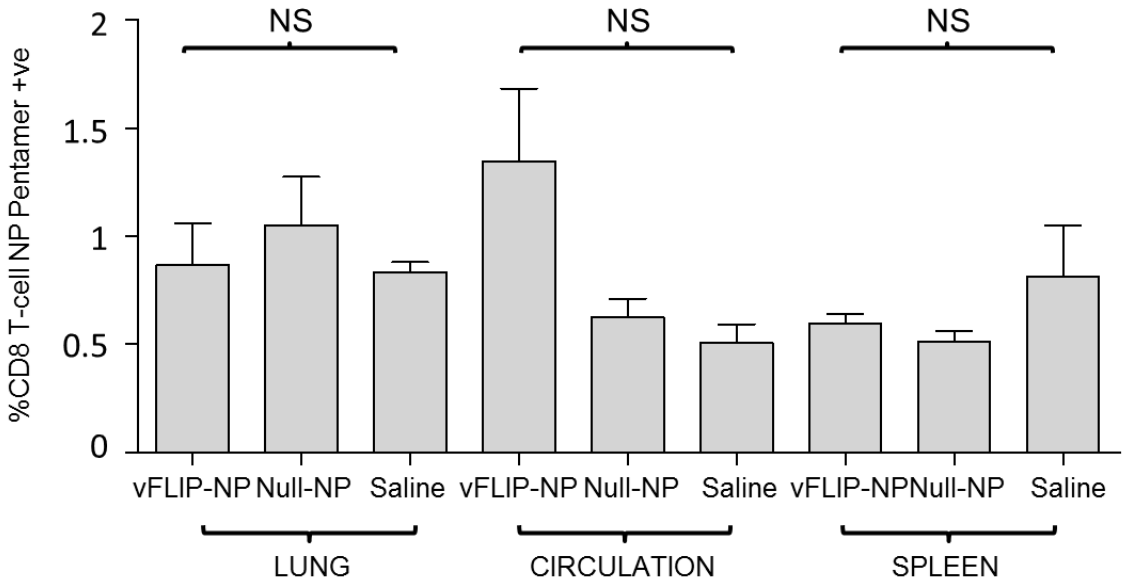


Figure 4-1 Intranasally-administered LV fail to prime detectable NP-specific T cell responses in lung, circulation or spleen. Populations were compared with a Student's t-test.

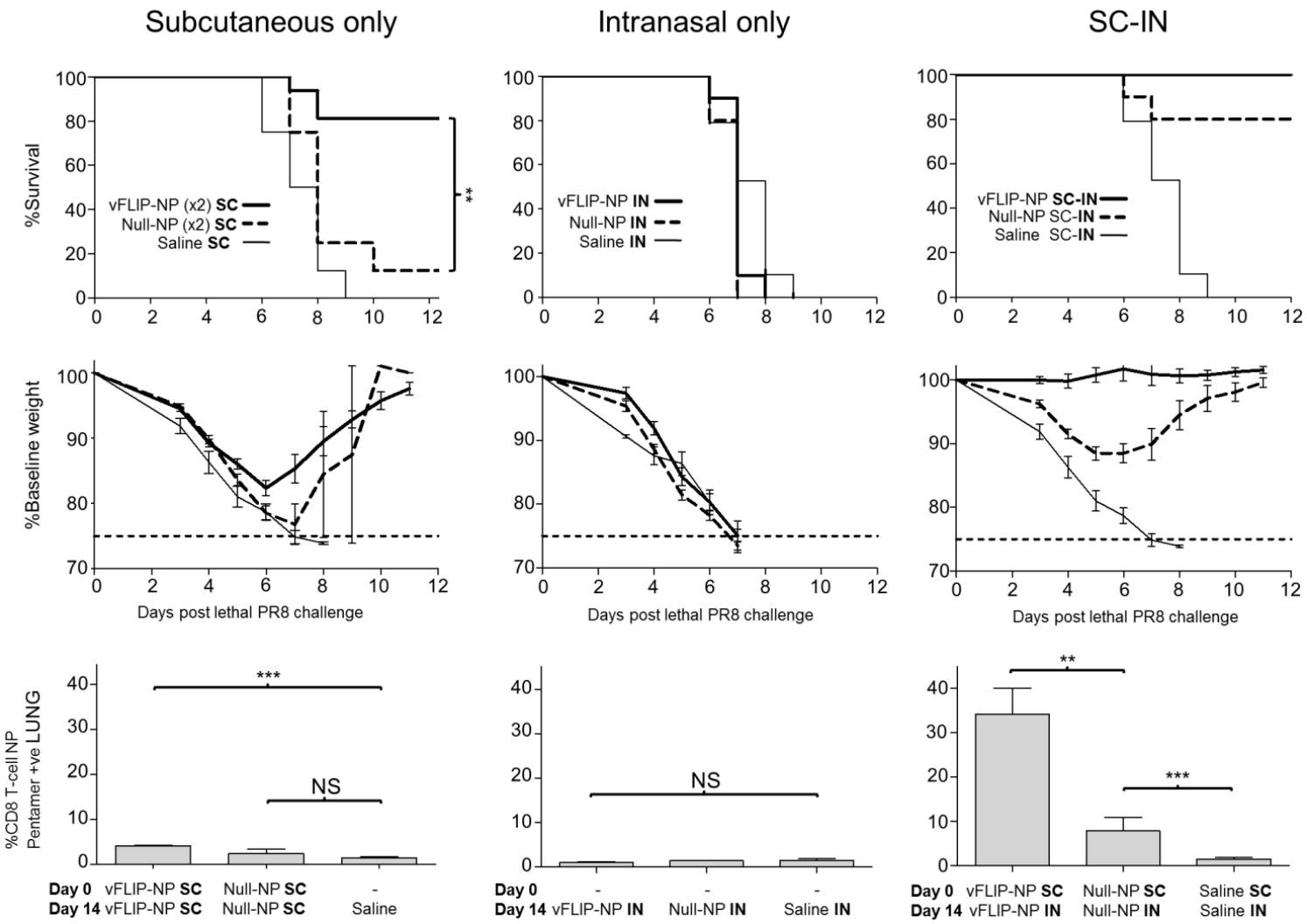


Figure 4-2 Response to subcutaneous, intranasal or SC followed by IN (SC-IN) immunisation. Mice were challenged with lethal A/PR/8/34 (PR8) or sacrificed for analysis 2 weeks after the final immunization of the regimen indicated on the x-axis. Top: Survival after lethal $2xLD_{50}$ challenge (n=10-18 per group). Both Mantel-Cox tests and Gehan-Breslow-Wilcoxon Test produced similar p-values for observed differences in survival. Middle: Weight loss from baseline after lethal PR8 challenge (n=10-18 per group). Average weights are taken from all survivors in the group at that time point. Bottom: Percentage of $NP_{147-155}$ pentamer positive CD8+ T cells from lung homogenate (n=4-6 per group).

4.3.3 Intranasal LV transduce AM with high efficiency

Influenza infection of AM is known to drive NFκB-dependent secretion of non-ELR CXC chemokines which preferentially attract mononuclear cells[440]. To compare activation of AM by influenza with NFκB activation by vFLIP, LV expressing vFLIP and GFP (vFLIP-GFP), or GFP alone (Null-GFP) or live PR8 influenza were administered intranasally to naïve BALB/c mice and bronchoalveolar lavage performed on day 4. FACS analyses of broncho-alveolar lavage (BAL) cells stained with anti-F4/80 and anti-CD11c are shown in Figure 4-3. These show efficient transduction (>75%) of $F4/80^{HI}CD11c^{HI}$ AM (histogram) with very high specificity (>99%, adjacent FACS plots). To investigate the possibility of transduction of parenchymal DC (which have interdigitating processes that access the lumen but would not be retrieved by BAL) we analysed total lung homogenate. Analysis of total lung cells by F4/80 and CD11c (Figure 4-4) revealed a similar profile to BAL, but with a greater number of interstitial or tissue macrophages (F4/80+ CD11c-) and DC (F4/80-, CD11c+). Again, AM were transduced with high specificity, with no detectable GFP+ DC, interstitial macrophages or "transitional" macrophages ($F4/80+CD11c^{INT}$).

4.3.4 NFκB activation by vFLIP leads to secretion of T cell chemoattractants but not up-regulation of co-stimulatory molecules.

NFκB activation increases transcription of a number of chemokine genes with NFκB elements in their promoters. Among these IP-10 (interferon-inducible protein of 10kd), MIP1α (macrophage inflammatory protein 1 α), MIP1β, MCP-1 (monocyte chemotactic protein-1), MCP-3 and RANTES (regulated upon activation, normal T cell expressed and secreted) play an essential role in T cell recruitment in response to influenza (reviewed in [441]). Intranasal administration of vFLIP-GFP, but not Null-GFP, resulted in greatly increased levels of these six chemokines in the BAL 4 days later (Figure 4-5). To determine whether these originated from transduced immune cells in the airway or

transduced epithelial cells, BAL cells were cultured for 4 days and then chemokines quantified in collected supernatants. FACS analysis of cultured cells confirmed more than 97% of the transduced (GFP+) population were AM ($F4/80^{HI}CD11c^{HI}$). Again, high levels of these chemokines were found in the supernatants of adherent cells from the vFLIP-GFP recipient group. Intranasal Null-GFP failed to generate a detectable chemokine response in BAL or in the supernatant of cultured adherent cells despite equivalent levels of AM transduction. Therefore vFLIP expression in AM stimulates secretion of quantities of T cell chemoattractants comparable to those released during influenza infection**.**

However, expression of CD80 or CD86 was not up-regulated in vFLIP-GFP transduced AM compared with Null-GFP or AM post influenza challenge. This is consistent with previous findings of impaired CD80 and CD86 expression in AM[393,394]. The ability of vFLIP transduced AM to secrete T cell chemoattractants upon NFκB activation but not up-regulate co-stimulatory molecules may account for both the proficiency of T cell recall and also failure of T cell priming by intranasal vFLIP-NP.

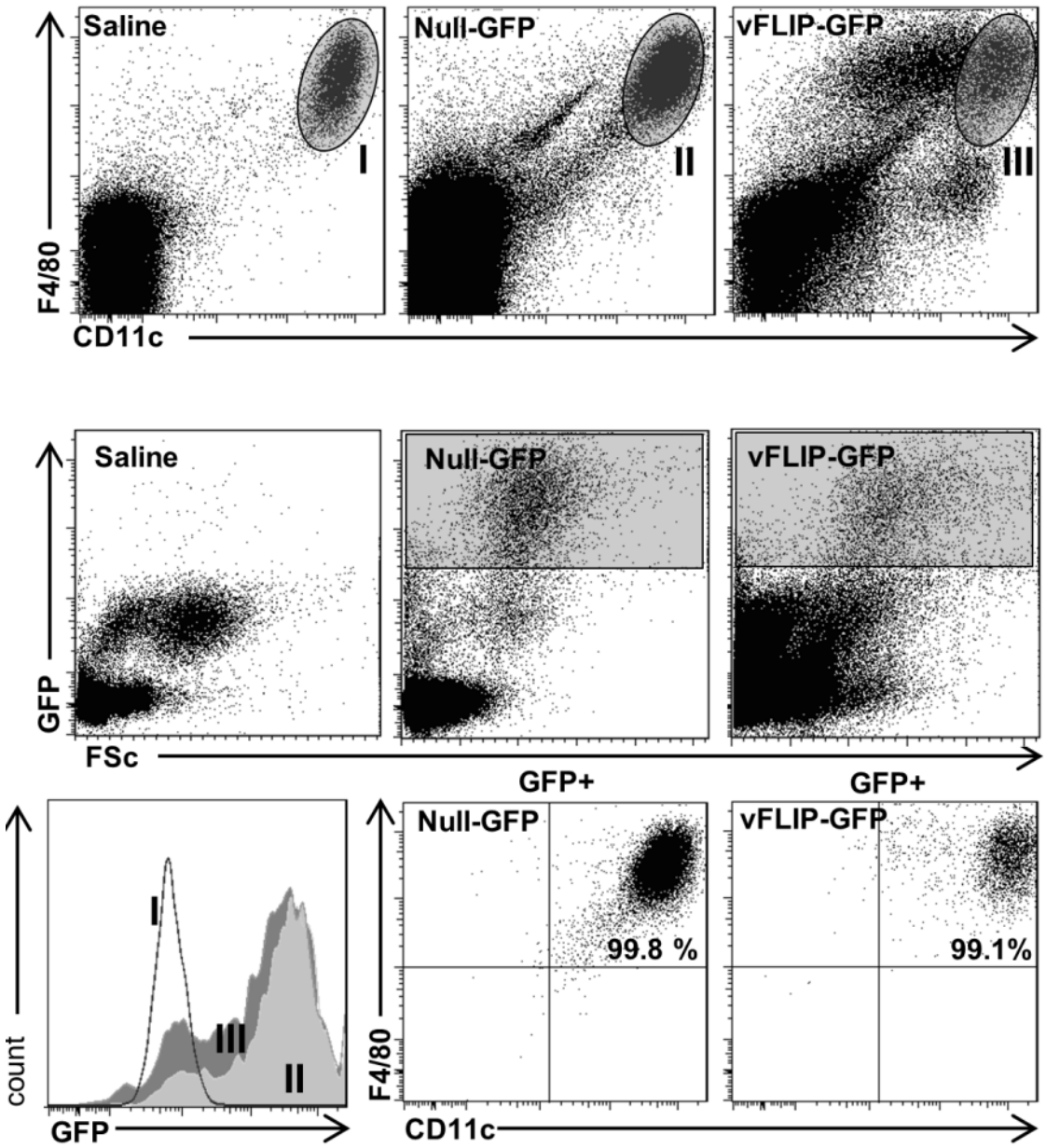


Figure 4-3 AM are transduced with high efficiency and specificity by LV. 200 ng (RT) of LV encoding vFLIP-GFP or Null-GFP (n=3 per group) were introduced intranasally. BAL was performed 4 days later and retrieved cells were stained for F4/80 and CD11c and analysed by FACS (top row). Circled areas indicate $F4/80^{HI}CD11c^{HI}$ AM populations in naïve, Null-GFP and vFLIP-GFP groups. Middle row shows gating of GFP+ cells for the bottom row plots. Histogram indicates transduction efficiency (>75%) as indicated by GFP signal in AM ($F4/80^{HI}CD11c^{HI}$) populations. Adjacent FACS plots (bottom) indicate LV transduce only $F4/80^{HI}CD11c^{HI}$ cells.

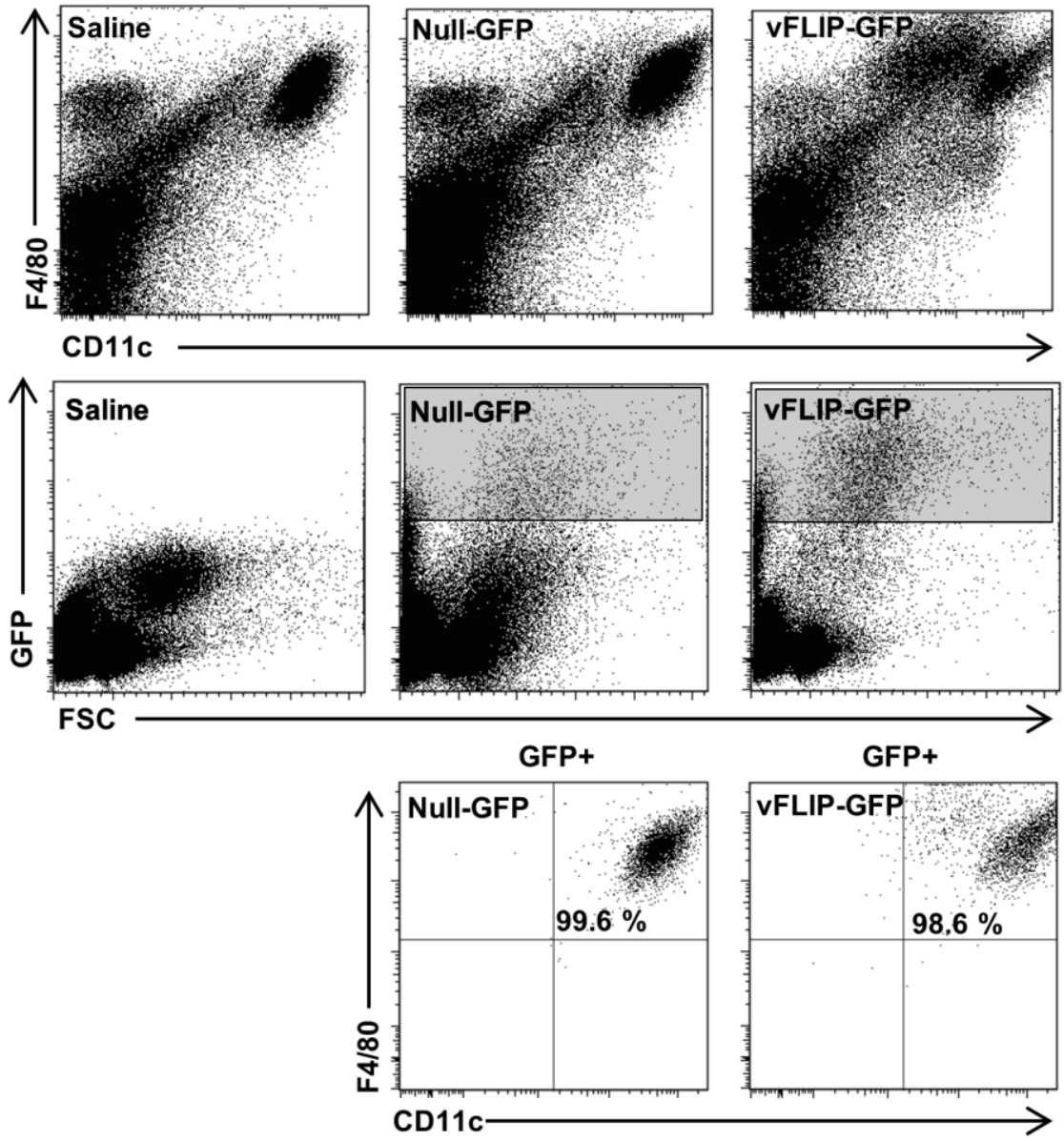


Figure 4-4 Analysis of total lung homogenate by the same method as in Figure 4-3. An additional population of F4/80^{+}CD11c^{-} (tissue macrophages) can be seen in the top row panels. These are not transduced by intranasal LV (bottom panel).

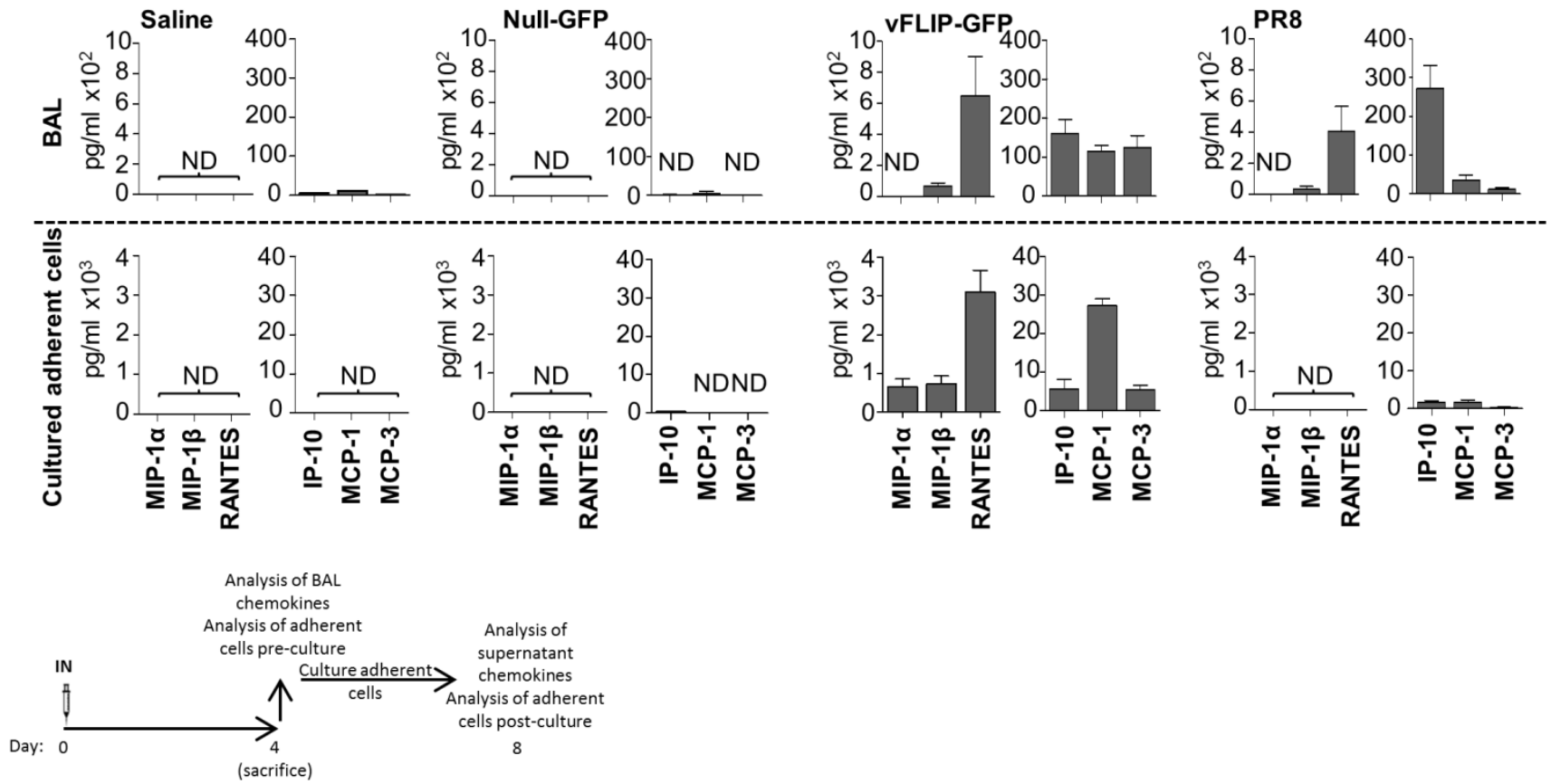


Figure 4-5 vFLIP transduced AM produce quantities of T cell chemo-attractants comparable mice infected with influenza A/PR/8/34. Concentrations of MIP-1α, MIP-1β, RANTES, IP-10, MCP-1 and MCP-3 were measured in a single 2ml BAL (n=5, top row) at day 4 after intranasal LV or influenza infection. These were also measured in 2 mL supernatants of adherent cells from BAL after 4 days culture *in vitro* (bottom row). FACS analysis of cultured adherent cells showed >97% of transduced cells (GFP positive) were F4/80^{HI}CD11c^{HI}. Transduced (GFP+) CD11cHI F4/80- DC were not detected by FACS before or after culture of BAL cells. ND= not detected.

4.3.5 Airway NP-specific CD8+ T cells generated by SC-IN vaccination are high in GzmB and Ki67 but cytokine responses are minimal

Substantial numbers of T cells were found in the airway by BAL 14 days after SC-IN vaccination: more than 60-fold greater than those found in unvaccinated mice. Numbers of CD8+ T cells were significantly higher in the vFLIP-NP SC-IN group versus the Null-NP SC-IN group, and nearly a third of total CD8+ T cells expressed high levels of GzmB without re-stimulation, indicating on-going cytotoxic potential (Figure 4-6). Over half (51%) of the NP-pentamer positive CD8+ T cells showed high GzmB expression in the vFLIP-NP SC-IN group compared with 30% of the NP-specific CD8+ T cells in the Null-NP SC-IN group ($p= 0.003$) and <1% in unvaccinated mice. CD4+ T cells were also recruited in large numbers to the airway by SC-IN vaccination, but there were no significant differences in numbers between vFLIP-NP and Null-NP groups. In both vFLIP-NP SC-IN and Null-NP SC-IN groups Ki67 staining was high in CD8+ and CD4+ T cells, indicating on-going proliferation in these populations. This contrasts with the observations of Woodland *et al* of airway T cell populations seen 2 weeks after primary infection which are non-replicating.

Re-stimulation of CD8+ and CD4+ T cells with class I and class II restricted NP peptides respectively elicited IFNγ, TNFα and IL-2 responses but these were seen in much lower percentages than GzmB staining (Figure 4-7). No significant differences in cytokine secretion were observed between vFLIP-NP and Null-NP SC-IN groups, with the exception of slightly higher IL-2 secretion seen upon restimualtion of CD8+ T cells in the vFLIP-NP group.

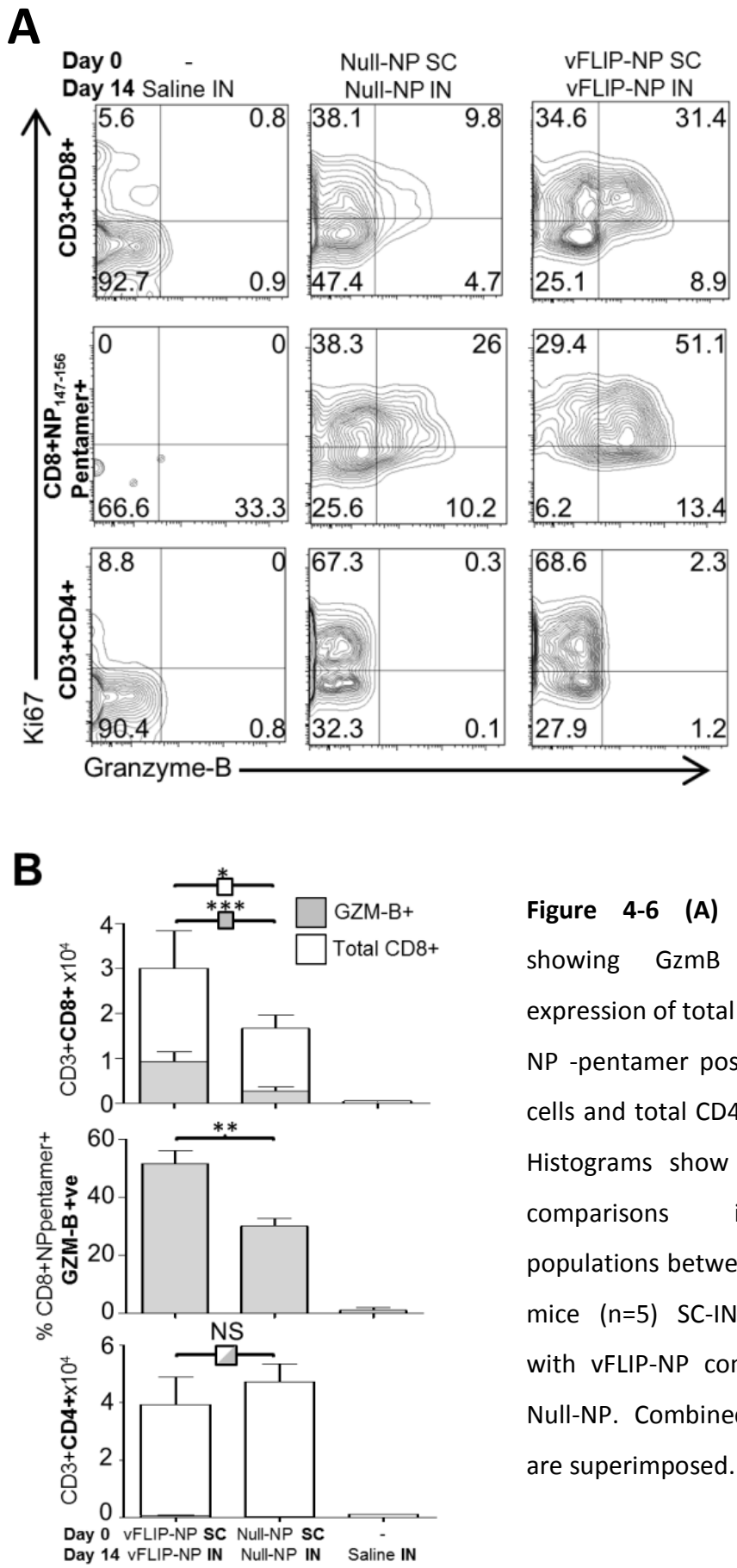


Figure 4-6 (A) FACS plots showing GzmB and Ki67 expression of total CD8+ T cells, NP -pentamer positive CD8+ T cells and total CD4+ T cells. **(B)** Histograms show quantitative comparisons in these populations between groups of mice (n=5) SC-IN immunised with vFLIP-NP compared with Null-NP. Combined bars (top) are superimposed.

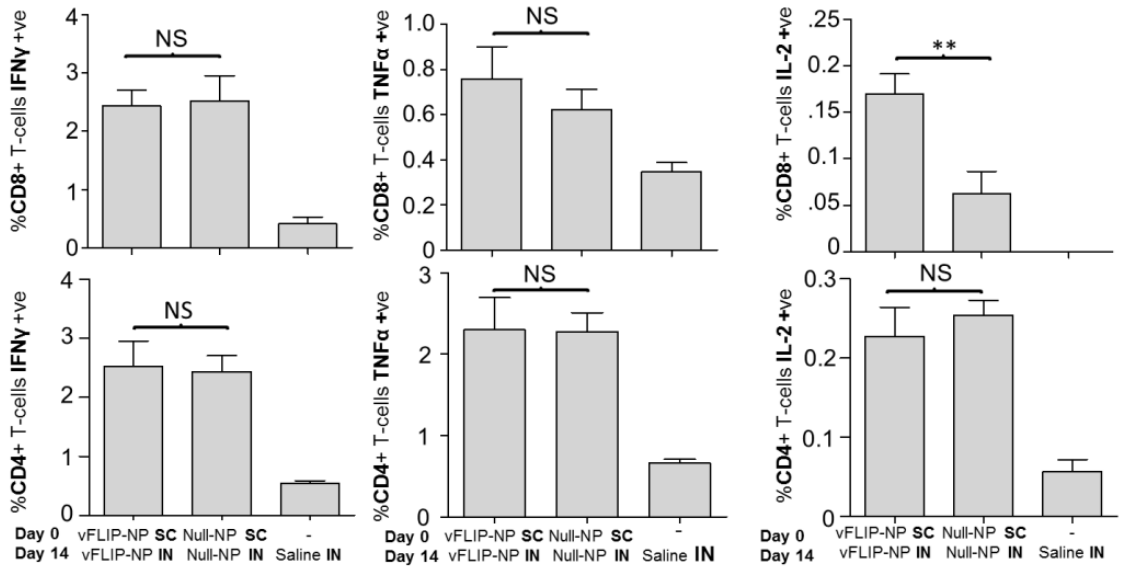


Figure 4-7 Intracellular cytokine staining of lung homogenate CD8+ (top) and CD4+ (bottom) T cells after SC-IN immunization. Mice were immunised subcutaneously then intranasally 2 weeks apart and sacrificed for analysis 2 weeks later. Mononuclear cells from lung (homogenized after exsanguination and removal of lymph nodes) were re-stimulated overnight *in vitro* with either MHC class I or II restricted peptides in the presence of brefeldin A prior to intracellular cytokine staining.

4.3.6 Both vFLIP and NP are required in intranasal lentiviral immunisation to maximize T cell recall and antigen-specific expansion respectively

We postulated that the major components of a recall response – chemotactic recruitment of memory cells and antigen-driven expansion – are principally instigated by transduction of AM with the components vFLIP and NP respectively, and therefore numbers of antigen-specific T cells would be diminished in either's absence. To test this we gave an intranasal LV expressing either vFLIP (vFLIP-GFP) or NP (Null-NP) to mice that had been immunised with vFLIP-NP subcutaneously two weeks earlier. The vFLIP-GFP intranasal recall increased total numbers of airway CD8+ T cells but the proportion of antigen-specific NP-positive CD8+ T cells remained similar to that seen in the circulation and spleen (Figure 4-8A). By contrast, Null-NP IN recall recruited less than half the number of total CD8+ T cells to the airway as vFLIP-GFP, but a greater proportion of these were NP-pentamer positive (18.4% vs. 5.2%, p=0.002). This is consistent with local antigen-driven expansion of NP-specific CD8+ T cells, corroborated by the finding of 6-fold higher numbers of Ki67+ CD8+$NP_{147\text{-}155}$ pentamer+ T cells in the airways of Null-NP IN recipients versus the vFLIP-GFP IN recall group. Both vFLIP-GFP and Null-NP IN recalls after vFLIP-NP SC priming generated similar absolute number of antigen-specific T cells in the airways but approximately three-fold lower than that seen with vFLIP-NP SC-IN recall. Correspondingly survival and weight loss after lethal influenza challenge were inferior to the complete protection observed with vFLIP-NP SC-IN immunisation (Figure 4-8B).

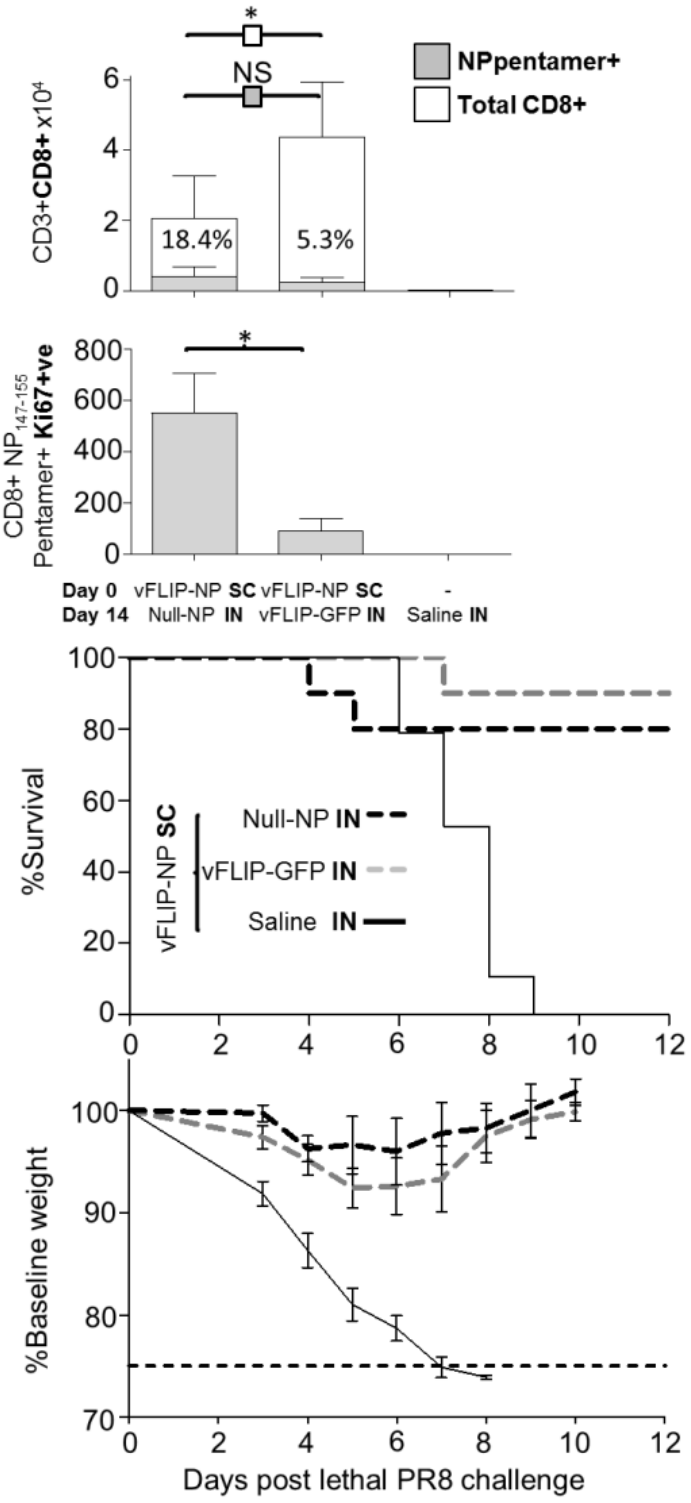


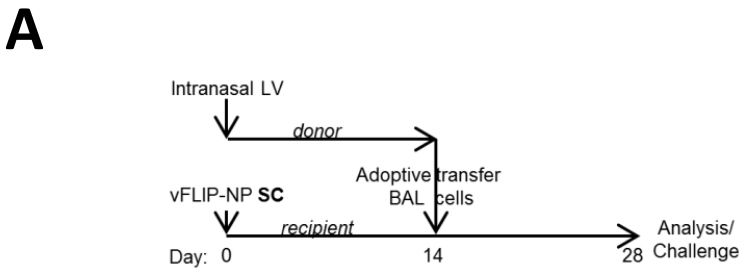
Figure 4-8 (A) Comparison of airway $NP_{147-155}$ pentamer+ CD8+ T cell numbers after SC vFLIP-NP immunization followed by intranasal vFLIP-GFP or Null-NP. Bars are superimposed. Number of Ki67+Pentamer+ CD8+ T cells in each group is shown in the lower panel. **(B)** Survival and weight loss after challenge with $2xLD_{50}$ A/PR/8/34.

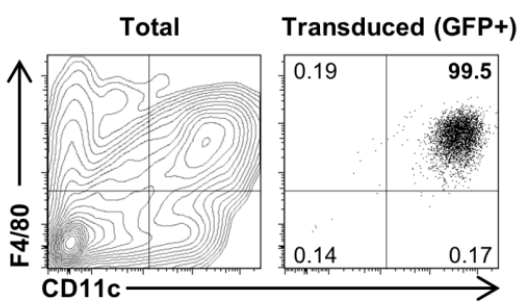
4.3.7 Adoptive transfer of transduced AM elicits lung T cell recall and protection against influenza

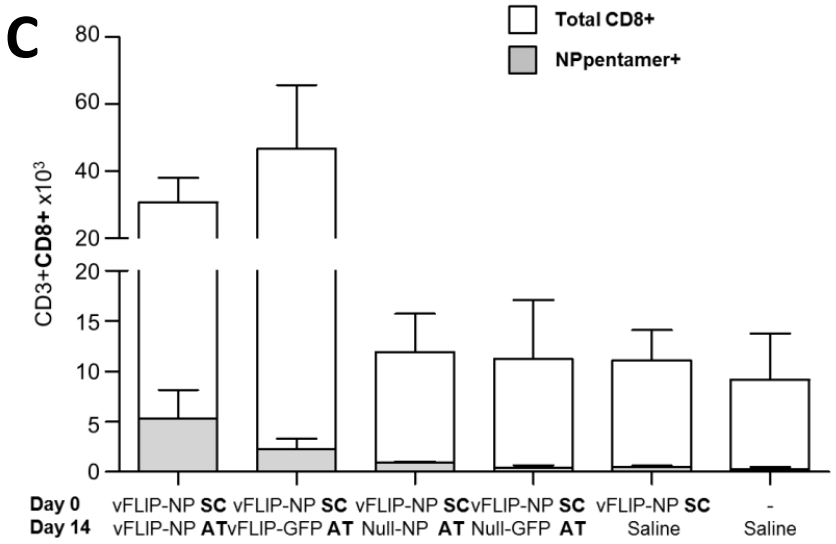
Whilst LV transduce AM with very high specificity amongst airway immune cells, they have also been reported to transduce approximately 10% of alveolar epithelial cells[442]. To determine whether AM alone are necessary to instigate T cell recall we attempted their depletion by clodronate liposomes[443] or diphtheria toxin in CD11c-DTR transgenic mice[444] prior to intranasal LV vaccination. However, both approaches were confounded by only partial AM depletion (58% and 60% depletion for clodronate and diphtheria toxin in the CD11c—DTR model respectively). Also, AM depletion resulted in infiltration of the airway by DC that would be susceptible to intranasal LV transduction and confound attempts to isolate AM as the agents of T cell recall. We also attempted to de-target expression of the transgene from AM by constructing an LV in which the transgene was followed 3' by 4 repeats of a target sequence for the haematopoetic-specific microRNA, miR-142-3p as previously described[318]. This leads to silencing of the transgene by microRNA binding to transgene mRNA which is then degraded. LV expressing 4-1BBL(h) as a surface marker, followed 3' by miR-142-3p repeats were delivered intranasally to 3 mice and expression of 4-1BBL on AM analysed after BAL 4 days later. The inclusion of MiR-142-3p did lead to a 50% reduction in expression of 4-1BBL, but this was nevertheless still 40 times greater than background levels, indicating incomplete silencing.

We therefore adoptively transferred AM transduced *in vivo* with intranasal LV into recipient mice that had been primed with SC vFLIP-NP two weeks previously (Figure 4-9). This approach takes advantage of the >99% specificity of AM transduction in the transferred population (Figure 4-3B), removing the need for cell sorting which incurs substantial processing losses of this highly adherent population. Two weeks after intranasal adoptive transfer, recipient mouse lungs were harvested from 3 mice per group for analysis of total lung T cells. This revealed a very similar pattern of T cell recall as direct intranasal administration of LV (Figure 4-2C). Whilst absolute numbers of $NP_{147\text{-}155}$ pentamer+ CD8+ T cells recalled by this means were 3-fold lower than achieved by direct intranasal LV, it is noteworthy that only 12,000 AM were

transferred, representing some 0.4% of the estimated total that would be transduced by direct IN LV administration[445]. Despite this, adoptive transfer of vFLIP-NP transduced donor AM induced a sufficient T cell recall response to confer 100% survival against lethal challenge with minimal weight loss (Figure 4-10).







	vFLIP-NP	vFLIP-GFP	Null-NP	Null-GFP	vFLIP SC	Saline
vFLIP-NP		0.104	**0.040***	**0.012***	**0.032***	**0.011 ***
vFLIP-GFP	0.245		0.088	**0.017***	**0.043***	**0.012 ***
Null-NP	**0.017***	**0.036***		**0.018***	**0.016***	**0.006 ***
Null-GFP	**0.011***	**0.015***	0.867		0.790	0.284
vFLIP SC	**0.013***	**0.033***	0.782	0.97		0.197
Saline	**0.005****	**0.011***	0.440	0.601	0.562	

Figure 4-9 Adoptive transfer of LV-transduced AM recalls large lung T cell populations. **(A)** Adoptive transfer schedule. **(B)** BAL cells from donor mice were washed and concentrated at 12,000 AM (F/480+CD11c+) cells per recipient mouse (40µl). FACS plots show high specificity of AM (F/480+CD11c+) transduction in donor cells. $NP_{147-155}$ pentamer positive CD8+ T cells were undetectable in BAL from donor mice. **(C)** CD8+ T cell populations from total lung homogenate of recipient mice 2 weeks after adoptive transfer. Comparative T-test p-values are given in the inset table. Bars are superimposed.

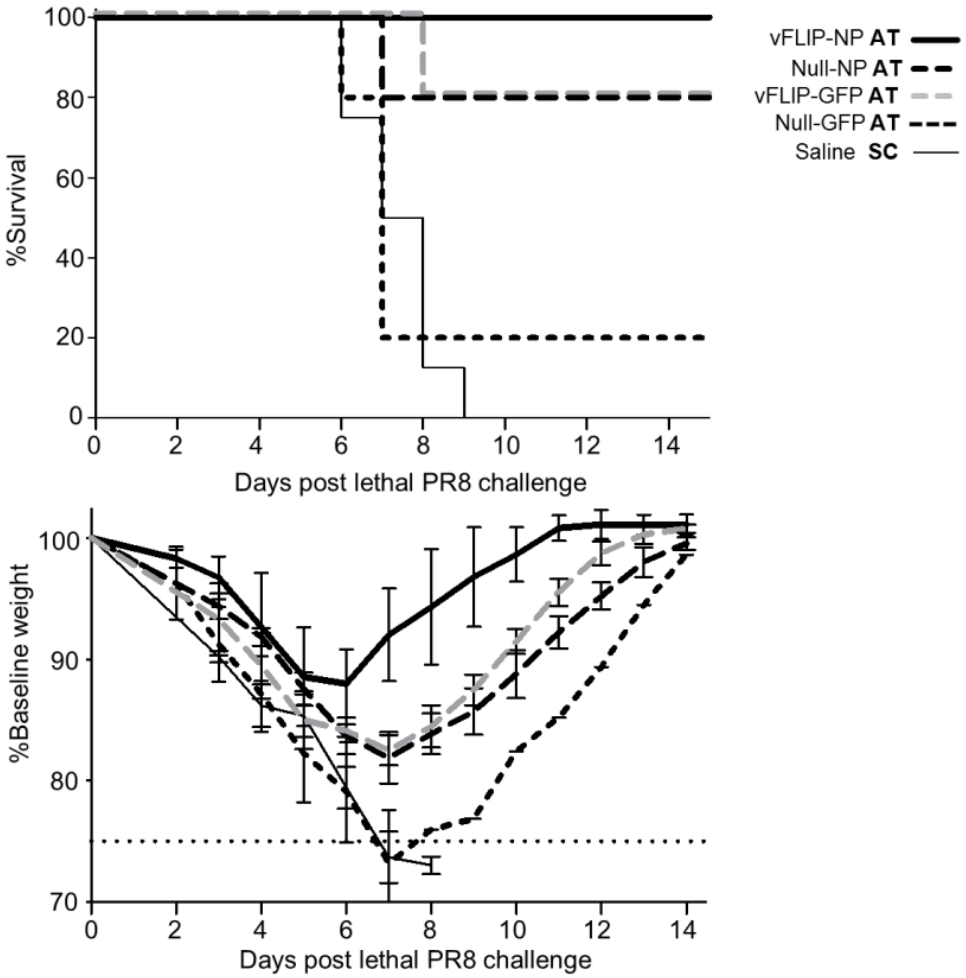


Figure 4-10 Survival (top) and weight loss in survivors (bottom) of recipient mice challenged 2 weeks after adoptive transfer as shown in Figure 4-9.

4.4 Summary

Intranasal LV fail to prime T cell responses to encoded antigen, but recall very large populations of antigen-specific T cells to the lung and airway in mice in which memory has been established by prior subcutaneous vaccination. SC-IN vaccination with vFLIP-NP generates sufficient lung-based populations of NP-specific CD8+ T cells to protect against lethal A/PR/8/34 challenge without weight loss or signs of clinical disease. AM are transduced with >75% efficiency and >99% specificity in BAL populations. Transduction of AM with vFLIP induces secretion of T cell chemoattractants but not up-regulation of co-stimulatory molecules. Examination of airway CD8+ T cells from SC-IN vaccinated mice reveal these are high in Ki67 and GzmB, indicating on-going proliferation and cytotoxic potential. vFLIP-NP SC-IN vaccination resulted in higher GzmB staining than Null-NP SC-IN vaccination, although both vFLIP-NP and Null-NP were broadly equivalent in generating cytokine responsive CD4+ and CD8+ T cells. Both vFLIP and NP are required to maximise recall and expansion of NP-specific CD8+ T cells in the airway. Adoptive transfer of transduced AM to subcutaneously primed mice recalls NP-specific T cells to the lung in a similar manner to directly administered intranasal LV.

4.5 Discussion

It is common in dendritic cell biology for activation to be measured in terms of expression of the 3 signals eponymously hypothesised to be required for T cell activation. States of macrophage activation are not so straightforwardly classified, partly because they exhibit significant phenotypic plasticity but also because they have essential functions beyond their immune effector role. For example, they have a key role as phagocytes, clearing the interstitial environment of effete or apoptosis cells and debris, with little or no release of immune mediators in the absence of “danger“ signals[446]. In the presence of these, for example upon phagocytosis of necrotic tissue containing HSPs, histones, DNA and nuclear proteins, macrophages can undergo substantial phenotypic changes and become efficient immune effector cells

coordinating an adaptive T cell response. However, they can also shift to regulatory or "alternative" states, which are poorly understood.

Mirroring the differentiation of T helper cells observed in the 1990s, there were parallel efforts to classify macrophages into either M1 or M2 phenotypes; the former designating classically activated macrophages and the latter "alternatively activated". Early versions of this paradigm held that classical activation occurs under the influence of TH-1 cytokines (such as TNFα) or LPS and results in IL-12-secreting macrophages capable of stimulating CD8+ and TH-1 CD4+ T cell responses and secreting pro-inflammatory cytokines such as IL-1 and IL-6. Alternative activation occurs under the influence of TH-2 cytokines such as IL-4 and IL-13, and results in an immunoregulatory phenotype of macrophage secreting IL-10 and supporting TH-2 skewed CD4+ T -cell responses[447].

NFκB signalling plays a central role in classical activation of macrophages, a final common pathway of both TNF α and LPS signalling. We therefore anticipated that NFκB stimulation may subvert the default immunoregulatory role of AM and generate classically activated APCs capable of T cell priming. However, intranasal vFLIP-NP failed to prime detectable T cell responses in the airway, lung, circulation or spleen suggesting NFκB activation alone is insufficient to generate AM capable of T –cell priming. Our finding of a lack of co-stimulatory molecule expression by AM despite NFκB activation suggests a fundamental limitation in T cell priming ability that occurs upon differentiation into AM from their interstitial or circulating monocyte progenitors. The signals and mechanisms governing this differentiation are poorly understood. AM exhibit high levels of CD11c, not seen in other macrophage types. This phenotype can be induced in non-pulmonary macrophages by transfer to the airway[448], with high GM-CSF and surfactant protein D (SP-D) concentrations driving differentiation. Faulty GM-CSF signalling (either genetically determined or a consequence of auto-antibodies against the GM-CSF receptor) is the molecular mechanism underlying pulmonary alveolar proteinosis (PAP), wherein AM are incompletely differentiated and demonstrate numerous impairments in surfactant catabolism, phagocytosis and intracellular bacteria killing[449]. However, the immunostimulatory function of AM is further impaired, rather than enhanced, in PAP.

This suggests other GM-CSF independent pathways turn off the ability of macrophages to prime T cell responses upon differentiation into AM.

Whilst poor at T cell priming, AM nevertheless play a key role as early detectors of danger signals and initiators of innate activation in the lung. This is particularly evident in influenza infection, wherein influenza-infected AM undergo rapid activation of NFκB (within 2 hours) and subsequently secrete chemokines. A striking pattern of AM chemokine secretion is observed, wherein secretion of neutrophil chemotactic substances are suppressed and secretion of mononuclear cell chemokines, such as RANTES, MCP-1, IP-10 and MIP-1α, are substantially increased[450]. The actions of these chemokines are not limited to T cells and also recruit dendritic cells into the airway, permitting competent priming of T cells in local bronchial associated lymphoid tissue and lymph nodes. In an activated state, AM can thus indirectly support T cell priming via DC recruitment to the airway.

We therefore endeavoured to phenotype AM after LV transduction according to their profile of chemokine secretion. This revealed similarities between the profile of chemokine secretion by vFLIP-GFP transduced AM and that seen in A/PR/8/34 infection. These T cell chemoattractants were also detectable in the supernatants of adherent cells after 4 days in culture. Analysis of these adherent cells before and after culture confirmed >99% specificity for transduction of F/480+CD11c+ cells, suggesting transduced AM rather than any transduced epithelial cells are the principal source of these chemokines. By contrast, influenza-infected adherent cells from BAL failed to produce significant quantities of chemokines during ex-vivo culture. This may indicate that AM are not the principal source of these chemokines following influenza infection (alveolar epithelial cells can produce IP-10 in response to influenza infection, for example) but a more likely explanation is that chemokine production by AM after influenza infection is transient, falling off rapidly in the absence of replicating virus. Indeed, *in vitro* studies of AM stimulation with influenza suggest chemokine production peaks under 24hrs post-exposure[451].

Despite the rapid response to influenza infection observed in AM *in vitro*, *in vivo* there is a 48 hour delay after influenza infection before innate activation – as measured by accumulation of type-I interferons, MIP-3α, MCP-1, IL-6 and TNFα– is readily

detectable. This has been described as a "stealth phase", attributed to influenza mediated inhibition of APC activation through NS-1[452]. It has also previously been proposed that pro-inflammatory signals from the innate immune system, epithelial and endothelial cells after infection in the lung may play a role in enhancing airway defense. CD11c-expressing cells are maintained in the lung and display enhanced antigen presentation for several months after influenza or RSV antigen is undetectable[301,302]. This may be the consequence of IFNγ secretion by γδ T cells recruited late in infection resolution[303]. AM are also sustained in an activated state following infection by high GM-CSF concentrations, thought to be secreted by both mesenchymal cells and memory T cells after infection[305]. This cytokine also recruits a new population of $CD11b^{HI}$ macrophages which may persist for many weeks within the airways[306]. These mechanisms of "innate imprinting" following infection appear to confer a degree of protection over subsequent infectious challenge independently of antigen-specific T cell responses. For example, prior administration of a modified heat-labile toxin from *E. Coli* (LTK63) to the lung enhances immunity to respiratory syncytial virus, influenza and Cryptococcus neoformans[453]. It is therefore striking that in addition to failing to prime T cell responses, AM transduced with vFLIP also confer no independent protection against influenza challenge, despite the "head-start" in sustained innate activation this appears to confer. Our results would suggest if there is a "stealth phase" of influenza infection in which activation of infected AM is delayed, it does not seem to confer an obvious viral advantage since pre-activation of NFκB in AM with vFLIP fails to confer a survival benefit.

DC are known to migrate into the airway and lung parenchyma during influenza infection shortly after an initial phase of DC migration in the opposite direction from lung to lymph nodes where the T cell response is initiated. The origin of these DC is unknown, but lung and airway-resident DC during influenza infection appear to be essential to the on-going local primary T cell response. In the lung parenchyma it is possible these DC establish BALT where naïve T cell-DC interactions are known to occur[454]. However, the majority of these lung-migrating DC are observed in the alveolar interstitium and airway, where they are thought to maintain and enhance T cell responses to influenza by increasing T cell migration to the site of infection,

inducing subsequent T cell proliferation and protecting T cells from apoptosis through on-going costimulation[455]. Figure 4-3 shows that vFLIP-GFP transduction recruits a population of DC into the airway ($CD11C^{HI}F4/80^{LO}$) and a population of "transitional" macrophages with CD11c expression somewhere between that of interstitial macrophages and AM. We envisaged that these would accelerate the process of influenza antigen uptake and presentation after migration to lymph nodes and through cross-presentation sustain and enhance the subsequent primary T cell response, shortening the duration of infection. However, intranasal vaccination with vFLIP-NP alone not only fails to prime an NP-specific T cell response but also does not appear to accelerate the primary response to subsequent infection, conferring no benefit upon survival or weight loss. Indeed, it is possible that the monocyte chemokine signal from vFLIP-activated AM in fact hinder retrograde migration of DC to local lymph nodes preventing efficient T cell priming.

In contrast with the absence of NP-specific T cell response and protection seen with intranasal administration, SC-IN vaccination with vFLIP-NP induces large numbers of NP-specific CD8+ T cells, representing in excess of one third of the total found in total lung homogenate and protects against lethal influenza challenge without clinical disease. This compares favourably with previous attempts to generate mucosal T cell immunity against influenza as mentioned in the introduction, such as the ITS nanoparticle and NP-M2 adenovector vaccine which generate 17% and 12.5% of antigen-specific T cells in lung homogenate respectively. Neither of these formulations have generated protection without weight-loss in the context of a lethal challenge with a highly pathogenic strain.

The efficacy of the vFLIP-NP SC-IN regimen in generating lung-resident T cells may be attributed to both the strong chemotactic signal generated by vFLIP activation of macrophages and the presence of NP driving antigen-dependent T cell proliferation. In either's absence, the number of NP-specific T cells found in lung-homogenate, and corresponding protection, is diminished. However, the high degree of protection induced by vFLIP-NP may not only be a function of the higher numbers of lung-and airway-based NP-specific CD8+ T cells generated by this approach, but may also be due to their functional phenotype. We have identified $GzmB^{HI}KI67^{HI}$ CD8+ T cells in the

airways 2 weeks after vFLIP-NP SC-IN vaccination, suggesting on-going cytotoxic activity and proliferation. Relatively small proportions of both CD4+ and CD8+ T cells in this compartment produce cytokines upon re-stimulation with their respective class II or I restricted NP peptides. This phenotype differs substantially from that observed in airway CD8+ T cells found in the airway following respiratory virus infection. These have been extensively characterised in a number of studies[456–460] which have consistently shown these are unable to proliferate in response to infection and are non-cytolytic, yet they are capable of rapid cytokine secretion on reencounter of antigen which may contribute to heterosubtypic protection[461,462]. Indeed, cytokine-secreting influenza-specific CD8+ T cells can be generated in very high numbers by repeated infection. Christensen *et al* used a similar systemic prime-intranasal boost strategy using intraperitoneal A/PR/8/34 infection followed by live intranasal X31 challenge (an H3N2 subtype containing an identical NP as PR8)[463]. This generated in excess of 70% of NP-specific T cells in BAL, with similarly high frequencies of IFNγ secretion on re-stimulation. Upon a lethal H7N7 challenge, these accelerated viral clearance and conferred a survival benefit but did not prevent clinical disease. At the time of writing, Lambe *et al* have shown that systemic (intramuscular) prime with an adenovector expressing NP+M1 boosted with intranasal MVA expressing the same antigens resulted in high levels of IFN-γ secreting CD8+ T cells in mouse airways (around 25%)[464]. Curiously this conferred little benefit in terms of weight loss or survival upon A/PR/8/34 challenge compared with unvaccinated controls. Thus the presence of high numbers of NP-specific IFNγ-secreting CD8+ T cells may not confer equivalent protection to the $GzmB^{HI}$ $IFN\text{-}\gamma^{LO}$ phenotype generated by SC-IN vFLIP-NP.

A simple explanation for these observed phenotypic differences would be the persistence of antigen in in the airway of SC-IN vaccinated mice but not after influenza infection or MVA vaccination, since both GzmB expression and also proliferation in effector CD8+ T cells are antigen-dependent. Some studies have demonstrated detectable processed antigen presentation for up to two months after influenza virus clearance, but this is restricted to DC in draining lymph nodes[465–467]. Chemokine secretion and antigen presentation by influenza-infected airway AM is short-lived since in addition to NFκB activation, infection initiates apoptosis in AM leading to cell death within 20-30 hours[468]. LV-transduced AM, by contrast, have been shown to persist for

the lifespan of the adult mouse[469]. The longevity of T cell populations generated by SC-IN LV vaccination is explored further in the next chapter.

Whilst we have shown here that LV-transduced AM are sufficient for T cell recall to the lung by adoptive transfer to SC primed recipient mice, we have not demonstrated that they are necessary. Intranasal VSV-G pseudotyped LV also transduce a small (~10%) proportion of alveolar epithelial cells which may also be agents of T cell recall. Depleting AM to a degree that would demonstrate their necessity for T cell recall would be challenging, since we have shown here that transfer of just 0.4% of the estimated total AM number is sufficient to induce T cell recall in subcutaneously primed recipient mice. Thus depletion would have to be near total to reliably exclude a role for epithelial cells in T cell recall. We found only partial depletion (~60%) could be achieved using intranasal clodronate liposomes or administration of diphtheria toxin to CD11c-DTR mice (in which the diphtheria toxin receptor is driven by the CD11c promoter). In both cases DC were recruited to the airway, making them susceptible to transduction by intranasal LV and confounding the isolation of AM as the agents of T cell recall. Furthermore, both transduced and untransduced AM are depleted by this technique, altering the immunoregulatory environment by removing inactivated AM. An alternative approach is to use an LV for T cell recall that co-expresses the diphtheria toxin receptor. Our early data (not shown) with a DTR-GFP LV shows complete, selective depletion of transduced AM and preservation of untransduced AM (without a consequent influx of dendritic cells) following intranasal diphtheria toxin administration. However, significant further optimisation of the LV is required to ensure sufficient and consistent expression of all three transgenes (DTR, antigen, and NFκB activator) from an F4/80 or CD11c promoter.

The ability to protect mice from a highly pathogenic strain at lethal doses, whilst preventing clinical disease, by targeting conserved T cell epitopes points towards significant potential for impact in the clinical domain. In this regard several key questions arise with regard to the safety, longevity and implementation of this approach in the context of cognate T cell memory as found in most of the adult human population. These are addressed in the following chapter.

5 Clinically relevant models of LV vaccination against influenza

5.1 Introduction

In this chapter we report an investigation of vFLIP-NP SC-IN vaccination in a series of models relevant to clinical application. These include:

- the impact of SC-IN LV vaccination on lung injury and viral load
- the duration of protection
- recall of naturally acquired T cell memory in mice and humans
- the impact of NP sequence variation on protection
- the efficacy of safer, non-integrating LV in vaccination.

5.1.1 Viral load, T cell response and lung injury

The ideal T cell vaccine against influenza would prevent clinical disease rather than merely limit its severity or duration. vFLIP-NP SC-IN vaccination prevents outward clinical signs of infection but this does not reveal its impact on T cell responses, viral load and lung injury during infectious challenge. As discussed in the introduction, current models of the effects of mucosal T cells are extrapolated from data derived from primary T cell responses in mice. These predict that any starting number of CD8+ T cells less than 10^5 would have little impact on the peak viral load or clearance time[213] because early in infection the number of susceptible cells remains high (due to an as yet insufficient type I IFN response) and the clearance of infected cells is overwhelmed by rapid viral spread. Accurately modelling the effects of pre-challenge mucosal T cells on peak viral titres and clearance time has translational relevance in two key regards. Firstly, the relative contribution of viral burden (and its direct cytopathic effects) and T cell response (especially pro-inflammatory cytokine production) to lung injury remains incompletely understood. For example, do very high T cell numbers inevitably lead to lung injury irrespective of the viral load, or vice-versa?

Secondly, the peak viral load and time to viral clearance is a key determinant of the spread of a new influenza strain throughout a susceptible population. The basic reproductive ratio, R(0), is a threshold parameter that estimates the number of secondary infections in a wholly susceptible population resulting from an infected individual. If R(0) is less than 1 the epidemic dies out, whereas if R(0) is greater than 1, the epidemic persists. R(0) is sensitive to both peak viral load and average duration of infection which both determine duration of viral shedding. Tracking the effects of SC-IN vaccination on influenza viral load after challenge thus has implications for the efficacy of this approach in limiting the spread of novel influenza strains in a population.

The ability to generate large numbers of NP-specific T cells in the lung prior to challenge provides an opportunity to clarify the relationship between lung injury and T cells and determine whether viral load is sufficiently controlled to impact upon the dynamics of influenza spread through a population.

5.1.2 Duration of protection

One of the major challenges in mucosal T cell vaccination is generating long-lasting populations of tissue-resident memory T cells that confer protection for the duration of an infectious threat. As described above, T cell mediated heterosubtypic immunity against influenza in mice wanes in parallel with falling numbers of lung-based T cells some 15 weeks after primary infection[234]. In humans, the duration of heterosubtypic immunity conferred by infection is less clear. Early studies by McMichael *et al* estimated circulating CD8+ T cell responses to NP peptides after infection to have a half-life of 18 months[233]. However, recent work by the same author highlights the marked discrepancies between T cell responses in the periphery and lung (as isolated by BAL) after influenza infection[470]. In the previous chapter we described $Ki67^{HI}$ CD8+ T and CD4+ T cells in the airway which differ from the non-replicative lung-resident T cells previously described after primary infection. This, together the previously reported longevity of LV transduced alveolar macrophages, raises the possibility that lung-based T cell populations may be sustained after SC-IN vFLIP-NP vaccination - by

on-going antigen presentation and a persistent chemokine signal – longer than is observed after primary infection. In this chapter we examine whether protection is sustained over a prolonged duration (4 months) and also whether T cell memory generated by SC vaccination can be effectively recalled by intranasal vaccination after a similarly prolonged delay.

5.1.3 Recall of naturally acquired T cell memory

The recent SOH1N1 pandemic illustrated the problems with timely delivery of an antibody-based vaccine in the face of a rapidly spreading strain. Given that indefinite mucosal T cell protection may be unachievable (or undesirable due to potential disruption of immunoregulatory homeostasis in the lung) a stockpiled universal vaccine that temporarily boosts mucosal immunity upon emergence of a new influenza reassortment strain may be a feasible approach to pandemic control. A prime-boost vaccine regimen has logistical drawbacks so recall with intranasal boosting of pre-existing influenza-specific T cells established by prior infection may be a more successful approach. To model this scenario we infected mice with A/Eng/195/09, a strain isolated from an individual with mild clinical disease during the recent pandemic, then attempted to recall memory T cells to the lung with intranasal vFLIP-NP.

5.1.4 Cross protection

The ability of T cell responses generated by vaccination against influenza NP to protect against multiple influenza subtypes is well established in mice. Emergent pandemics, however, frequently result from major reassortments from strains that were previously limited to non-human species which can include substantial variations in the conserved internal proteins. For example, the A/Eng/195/09 NP has only 91% amino acid homology with NP from A/PR/8/34. The degree to which a T cell response generated against one NP will protect against infection with a divergent sequence is likely to be determined by the degree of preservation of class I and class II restricted

epitopes. The latter differs between these strains. We therefore tested the ability of SC-IN vFLIP-NP, in which the NP is identical to A/PR/8/34 to protect against A/Eng/195/09.

5.1.5 Vaccine safety

Although lentiviral vectors are in use in clinical trials currently, safety concerns remain over the risk of insertional mutagenesis with use of integrating viral vectors. Clonal cell dysregulation after transduction with gamma retroviral vectors has led to leukaemias and myelodysplasias in several clinical trials[471,472]. Insertion of LTR elements next to proto-oncogenes appears to be the principal oncogenic mechanism in these cases. Mutagenic events following the use of LV based on HIV-1 in clinical trials, such as the treatment of X-linked adrenoleukodystrophy, have not been observed to date[473]. However, aberrant splicing events have been observed following the use of LV in humans, wherein cellular mRNA transcripts have been up-regulated following insertion into splice acceptor sites in the vector backbone[474].

One means of addressing this risk is through the use of integration-deficient lentiviral vectors. These have mutations in either the integrase, LTR or both. After cell entry and reverse transcription the vectors persist as circular DNA episomes. Homologous recombination within the two LTRs generates a circular episome with a single LTR, whereas less frequent non-homologous end-joining of the linear episome results in a circular episome with two adjacent LTRs. Since these have no origin of replication, expression is lost as the target cell divides but should persist in non-dividing cells such as neurones[475] and muscle cells[476]. Our group has previously demonstrated that nonintegrating lentiviral vaccines generate a humoral and cellular response against encoded antigen[126]. Negri *et al* have shown that human monocyte derived DC and macrophages transduced with integrase-defective LV expressing influenza M1 can expand autologous M1-specific CD8+ T cells[477].

However, expression of the LV transgene from circular episomes is as little as 1/10th of that seen with integrating LV[478,479]. This may not impede generation of primary CD8+ T

cell responses which are less sensitive to antigen quantity, but may impair secondary responses, wherein antigen quantity is a major determinant of the magnitude of CD8+ T cell recall. This is pertinent to their ability to recall existing T cell memory by an intranasal route which is tested in the experiments described in this chapter.

5.2 Aims

- To investigate the relationship between T cell number, viral load, cytokine response and lung injury during infection in mice with systemic CD8+ T cell immunity (SC vaccinated), mucosal T cell immunity (SC-IN vaccinated) and naïve mice

- To determine whether protection conferred by vFLIP-NP SC-IN vaccination is preserved for the duration of an influenza season (approximately 4 months)

- To determine whether T cell immunity generated by infection can be recalled by intranasal vFLIP-NP for protection, and whether human T cell memory can be recalled *in vitro* by autologous APCs transduced with LV

- To determine whether SC-IN vFLIP-NP vaccination confers protection against A/Eng/195 containing a non-homologous NP.

- To determine whether SC-IN vaccination with non-integrating LV can generate T cell responses against NP

5.3 Results

5.3.1 Intranasal vaccination limits influenza replication without lung injury or cytokine accumulation

Given that T cells are established mediators of inflammation during the influenza response, generating large populations of lung- and airway-based memory T cells by vFLIP-NP SC-IN immunisation has potential to enhance tissue injury upon challenge. We therefore examined the relationship between T cell number, secondary effector cytokine responses, viral load and lung injury after influenza infection in mice vaccinated with vFLIP-NP by SC or SC-IN regimens. Unimmunised mice (n=22) were challenged with a sub-lethal (0.8xLD_{50}) dose of A/PR/8/34. vFLIP-NP SC (n=20) group mice were immunized twice subcutaneously with vFLIP-NP 50ng 2 weeks apart then challenged with 2xLD_{50} of A/PR/8/34 2 weeks after the final immunization. vFLIP-NP SC-IN (n=18) mice were immunised with vFLIP-NP 50ng subcutaneously followed by intranasal vFLIP-NP 200ng 2 weeks later, then challenged with 2xLD_{50} of A/PR/8/34 2 weeks after the final immunization. In all groups 3 mice were analysed pre-challenge and on 3, 6, 9 and 15 days after challenge.

Figure 5-1B shows that there were marked differences in the ability of subcutaneous or vFLIP-NP SC-IN immunized mice to control viral replication. In the SC-IN group, the presence of effector phenotype ($CD62L_{LO}$) antigen-specific T cells in the airway and lung homogenate before challenge corresponded with rapid control of viral replication, which peaked 3 days sooner and at 14-fold lower levels than the subcutaneously immunised group. Notably, in the subcutaneously immunised group, NP-specific T cells did not start to accumulate in the lung and airway until 6 days after viral challenge. This corresponded with both the nadir in weight and peak viral load in lung homogenate. This response was nevertheless faster than the primary response in unvaccinated animals given a sub-lethal challenge, wherein numbers of effector phenotype $CD62L_{LO}$ NP-specific T cells in lung homogenate were 5-fold lower at this time-point.

Both T cell derived IFNγ and TNFα play a prominent role in lung injury[480,481]. In humans, strong and early CD4+ TH-17 responses have been associated with a more severe illness during the recent swine-origin H1N1 pandemic, and in knockout mouse models IL-17 has been shown to be essential for lung injury, weight loss and neutrophil infiltration but dispensable for viral clearance[273,274]. Similarly, IL-4 and IL-10 CD4+ T cell responses potentiate lung injury and airway hypersensitivity in influenza infection of mice without enhancing viral clearance or protection[272]. We therefore examined levels of these cytokines before and after influenza challenge in lung homogenate (Figure 5-1C and Figure 5-2B).

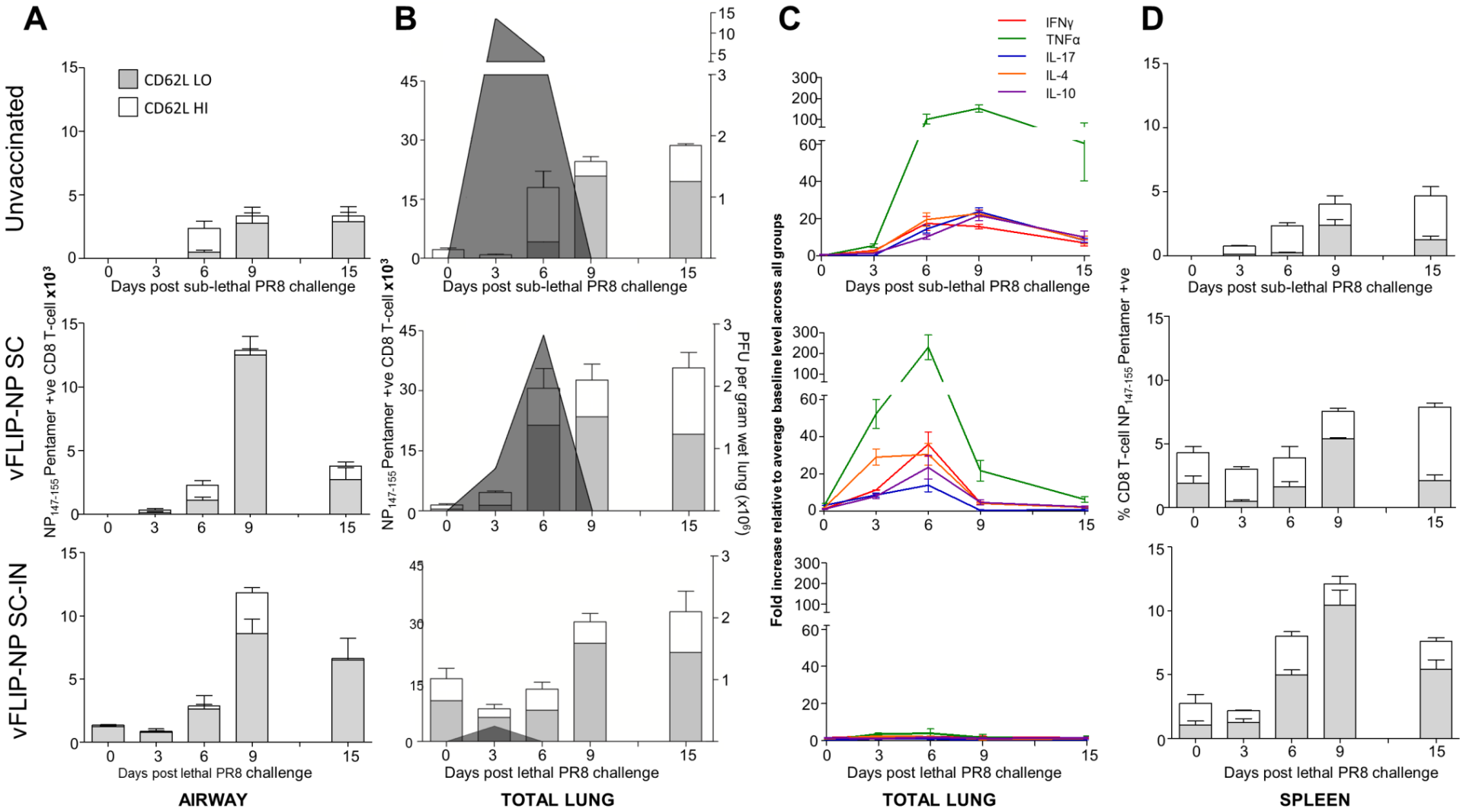

A
B
C
D
Unvaccinated
vFLIP-NP SC
vFLIP-NP SC-IN
CD62L LO
CD62L HI
IFNγ
TNFα
IL-17
IL-4
IL-10
Days post sub-lethal PR8 challenge
Days post lethal PR8 challenge
NP147-155 Pentamer +ve CD8 T-cell x10³
PFU per gram wet lung (x10⁶)
Fold increase relative to average baseline level across all groups
% CD8 T-cell NP147-155 Pentamer +ve
AIRWAY
TOTAL LUNG
TOTAL LUNG
SPLEEN

Figure 5-1 Airway and total lung CD8+ $NP_{147-155}$ pentamer+ T cell responses, viral loads and T cell cytokine burden after influenza challenge. Mice were immunised and challenged as described in the text (page 179) **(A)** CD8+ $NP_{147-155}$ pentamer+ T cell numbers in 2x2ml BAL per mouse. Stacked bars show CD62 low and high populations. **(B)** CD8+ $NP_{147-155}$ pentamer+ numbers in total lung homogenate. Overlaid polygon indicates viral load by plaque assay adjusted for wet lung mass. **(C)** Levels of T cell cytokines in lung homogenate (IFNγ, TNFα, IL-4, IL-10, IL-17) which have previously been implicated in lung damage during influenza infection. Levels are shown relative to the baseline (day 0) average across all three groups. **(D)** Splenic NP-pentamer responses during infection.

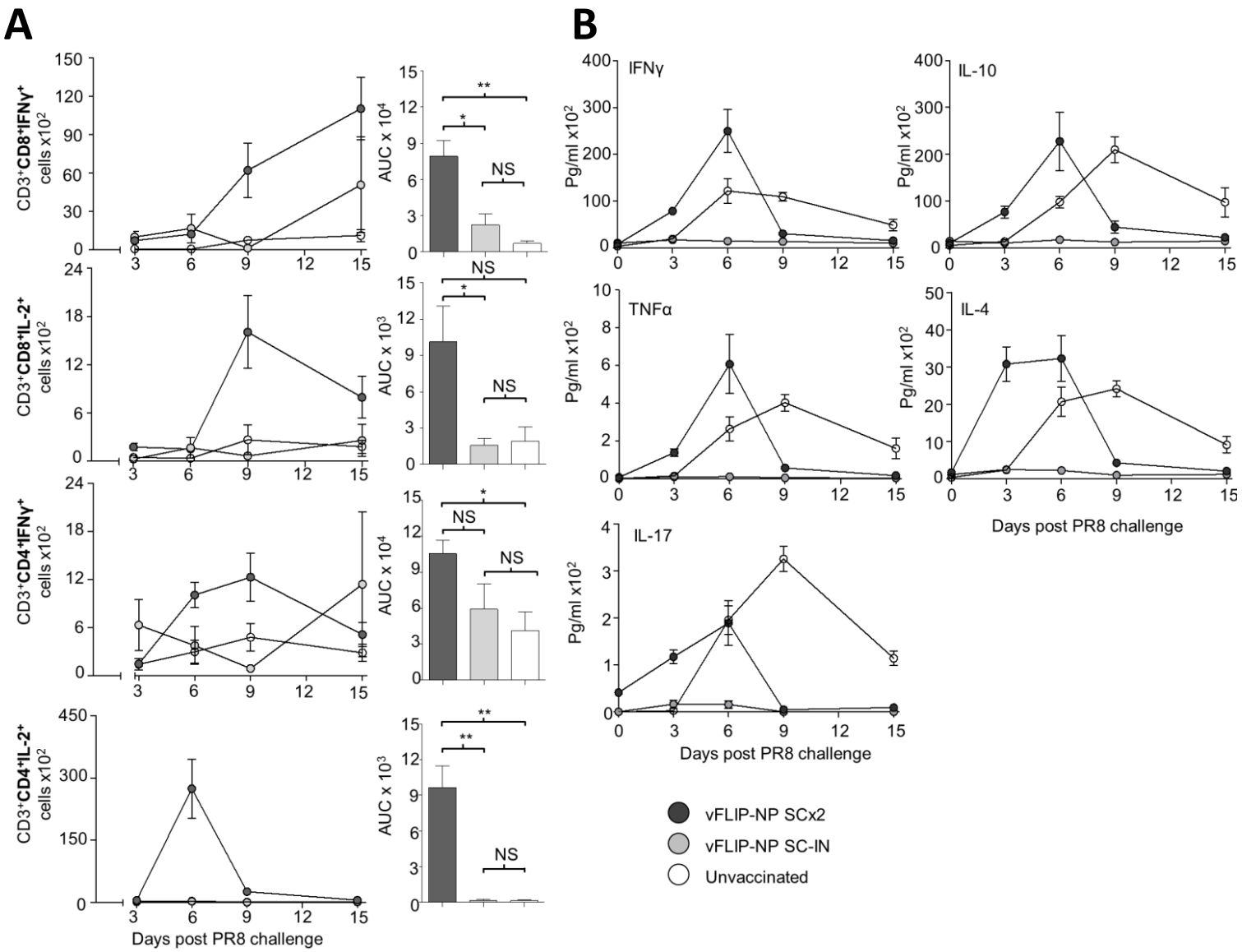


Figure 5-2. T cell and total cytokine responses in lung after influenza challenge. **(A)** Lung homogenate CD4+ and CD8+ T cell cytokine responses after PR8 challenge. Groups of mice were immunised as described in the main text. The adjacent histogram shows area-under the curve analysis for each immunization group over the 15-day study period. **(B)** Total cytokine levels (pg/ml) in lung homogenate post-influenza challenge from which fold-increase values in Figure 5-1 are derived. Error bars show +/-S.E.M.

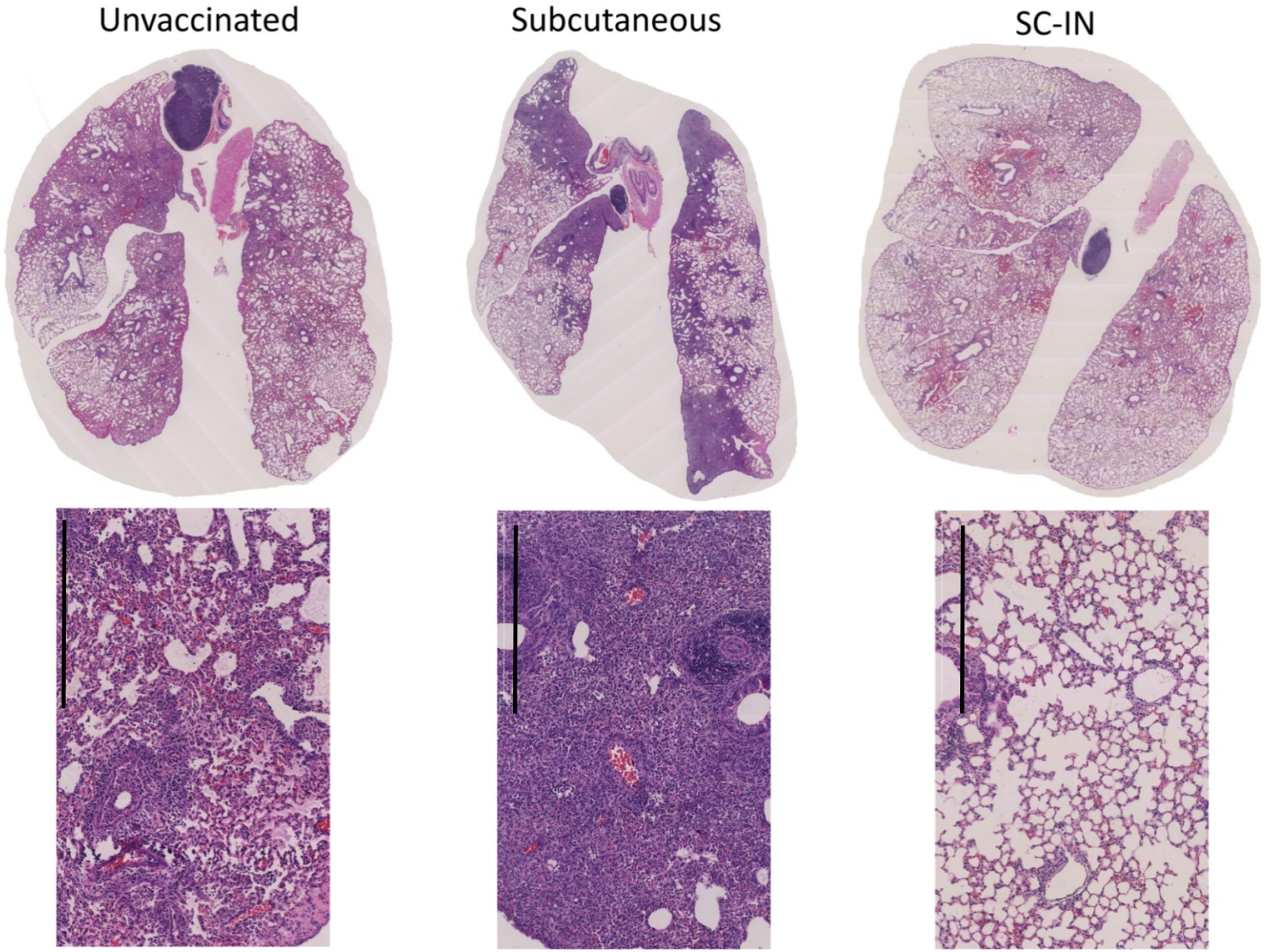


Figure 5-3 Histological analysis of lungs after influenza challenge. One mouse per immunization group (as described on page 179) was withdrawn at day 6 for histological analysis of lung tissue (without prior BAL). Coronal sections were stained with hemotoxylin and eosin prior to analysis. Inset box shows 10x magnification from the proximal right lower lobe of each mouse. Bar indicates 500 µm.

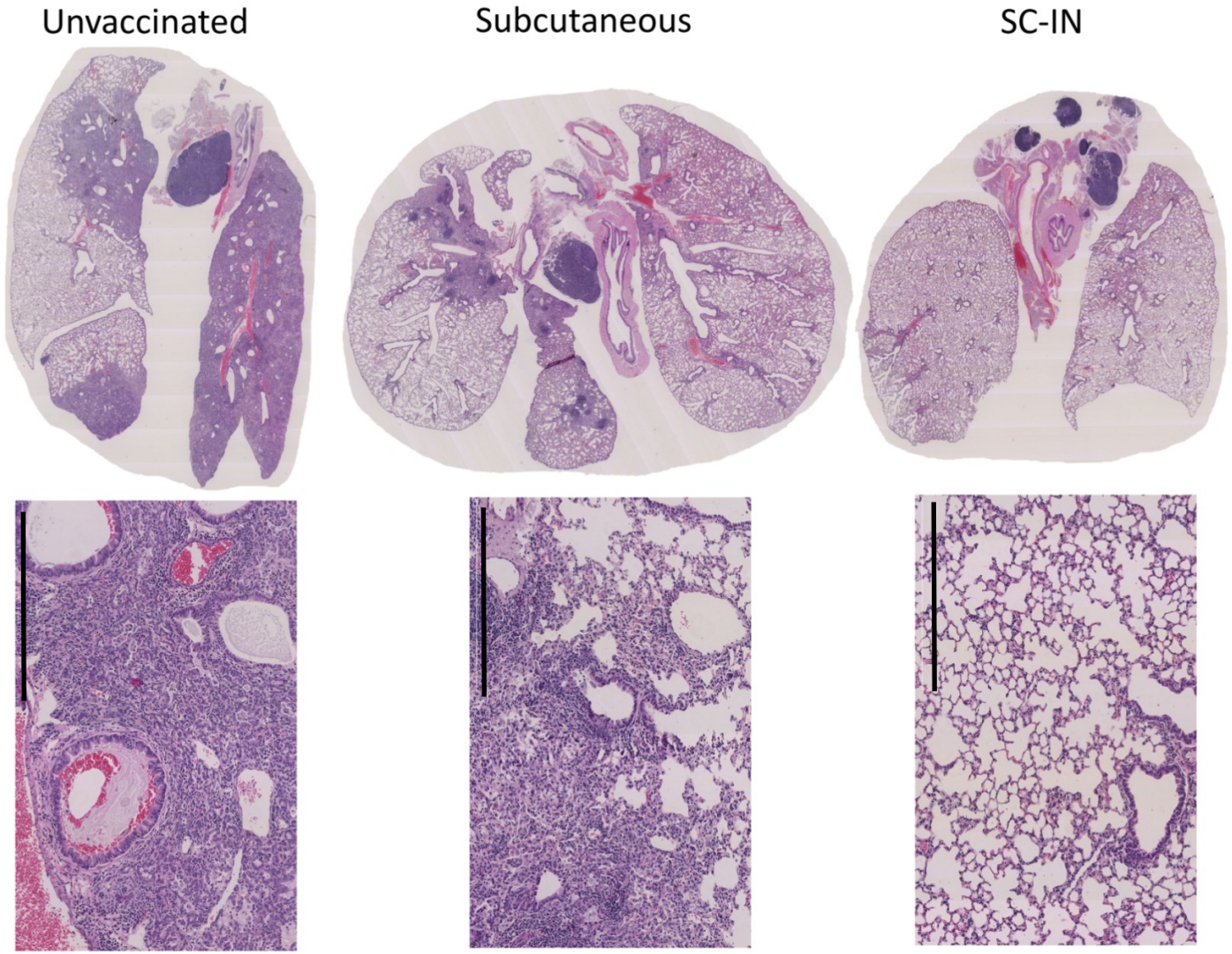


Figure 5-4 Histological analysis of one mouse per group withdrawn at day 15.

In naïve and subcutaneously vaccinated mice, levels of TNFα, IFNγ, IL-10, IL-4 and IL-17 rose in line with numbers of pulmonary NP-specific T cells and both reached significantly higher peaks in the subcutaneously vaccinated group, despite the higher peak viral titres in unvaccinated mice. However, in lung homogenates in the SC-IN group before and after challenge, these cytokines were minimal or absent (for absolute concentrations, see Figure 5-2). These differences corresponded to lung CD4+ and CD8+ T cell-specific IFNγ and IL-2 cytokine responses to overnight re-stimulation *in vitro*. Again, these were significantly greater and more sustained in SCx2 vFLIP-NP vaccinated mice than in SC-IN vFLIP-NP group or unimmunised mice by area-under the curve analysis (Figure 5-2A).

Histological analysis of lungs taken from one mouse in each group at days 6 and 15 revealed marked differences in the degree of lung injury (Figure 5-3 and Figure 5-4 respectively). In SC-IN vaccinated mice, although T cell infiltration was evident, no areas of airway loss or consolidation were found at day 6 or day 15. In SCx2 vaccinated mice however, there were multiple areas of consolidation in which the airway lumen was obliterated by inflammatory cell infiltrate, epithelial cell hyperplasia and haemorrhage. These changes persisted at day 15. This focal damage appeared more severe than that seen following influenza infection of naïve mice despite higher peak viral titres in the unvaccinated group.

5.3.2 Antigen-specific CD8+ T cell populations and protection against influenza established by intranasal vaccination are sustained for at least 4 months

Generating long-lived protection against influenza is critical to effective vaccination coverage during an influenza season or emerging pandemic. We therefore investigated whether lung T cell levels and protection would be sustained 4 months after SC-IN vFLIP-NP immunisation ("early recall" group). Whilst a clinically applicable vaccine would require rapid induction of mucosal immunity and thus a short interval between SC and IN immunizations, the antigen specific T cell population 2-weeks after subcutaneous vaccination is likely a mix of waning primary effectors and established memory T cells. In order to determine whether lung-based CD8+ T cells could be recalled from an established systemic memory population, we examined whether IN immunization with LV 4 months after SC vaccination could recall a memory-only T cell population ("late recall" group).

Analysis of mouse lung homogenate 4 months after SC-IN immunization ("Early recall", Figure 5-5) revealed no significant difference compared with total lung CD8+ T cell numbers in mice receiving vFLIP-NP IN 2 weeks before analysis ("late recall"). A large population of $NP_{147\text{-}155}$ pentamer+ CD8+ T cells remained 4 months after intranasal recall with vFLIP-NP IN ("early recall") which conferred complete protection from lethal influenza challenge. Similarly, the 4-month interval between subcutaneous prime and intranasal boost with vFLIP-NP had no effect on the level of T cell recall which was comparable to that resulting from the standard SC-IN 2 week interval in both quantity and degree of protection upon challenge.

5.3.3 SC-IN vFLIP-NP protects against A/Eng/195/09 and intranasal vaccination NP can boost naturally-acquired cross-strain immunity

Whilst the presence of memory T cells specific to influenza NP have been repeatedly shown to cross-protect against other subtypes sharing the same dominant T cell epitopes, a less examined question is whether this protection is preserved despite variation in NP sequence between strains. A/Eng/195/09 NP shares only 91% amino acid homology with A/PR/8/34 NP and the well-described H2-K^d-restricted CD4

epitope NP_{57-78} is non-identical. To determine whether the protection conferred by vFLIP-NP SC-IN vaccination was maintained despite this sequence variation, mice were challenged with $5x10^4$ PFU of A/Eng/195/09 and monitored for weight loss. Whereas naïve mice developed a prolonged illness losing approximately 10% of baseline weight, vFLIP-NP SC-IN vaccinated mice showed no signs of disease or weight loss (Figure 5-6B).

Much of the human population has had prior influenza exposure and thus has cognate T cell memory. A subcutaneous vaccination may be dispensable if naturally-acquired T cell memory can be boosted by intranasal vFLIP-NP administration. To model this, we infected naïve mice with non-lethal A/Eng/195/09, an H1N1 strain isolated from a patient in the UK during the recent swine origin H1N1 pandemic causing mild clinical disease, and then examined the effect of intranasal boosting with vFLIP-NP. Prior infection with A/Eng/195/09 gave 44% protection against lethal A/PR/8/34 challenge 64 days later, though all survivors lost considerable weight indicating the absence of cross-neutralizing antibodies against surface epitopes. However, vFLIP-NP intranasal boosting after non-lethal A/Eng/195/09 conferred 100% protection without weight loss against lethal A/PR/8/34 challenge (Figure 5-6A).

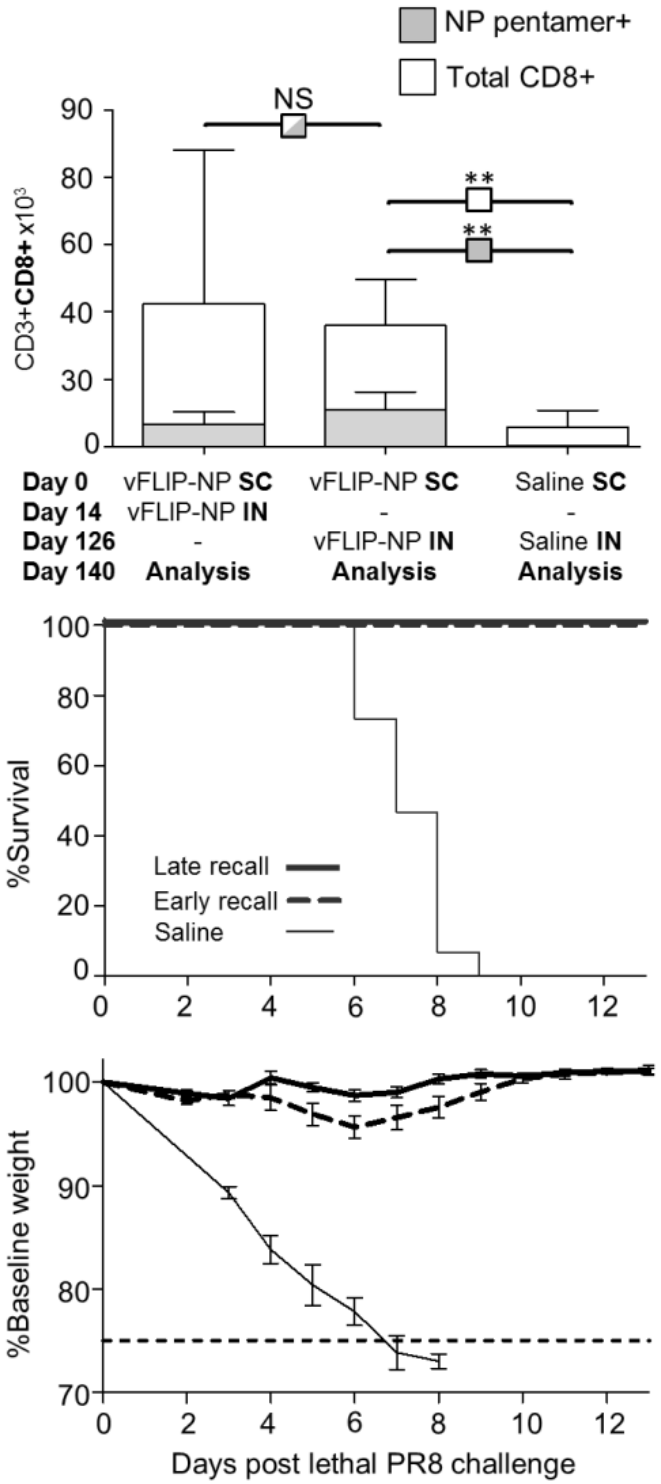


Figure 5-5 Longevity of SC-IN vFLIP-NP protection and application in clinically relevant models. Long-term preservation of lung-based T cell memory populations 4 months after vFLIP-NP SC-IN and protection against lethal $2xLD_{50}$ A/PR/8/34 challenge. Top: Lung total CD8 and superimposed $NP_{147-155}$ pentamer CD8+ T cells following intervals between subcutaneous and intranasal immunization and challenge indicated on the x-axis. Levels were compared using Students T test. All mice were challenged on day 140 (n=10 per group) or withdrawn for analysis (n=5 per group). Survival (middle) and weight loss in survivors (bottom) following challenge with lethal A/PR/8/24 are also shown.

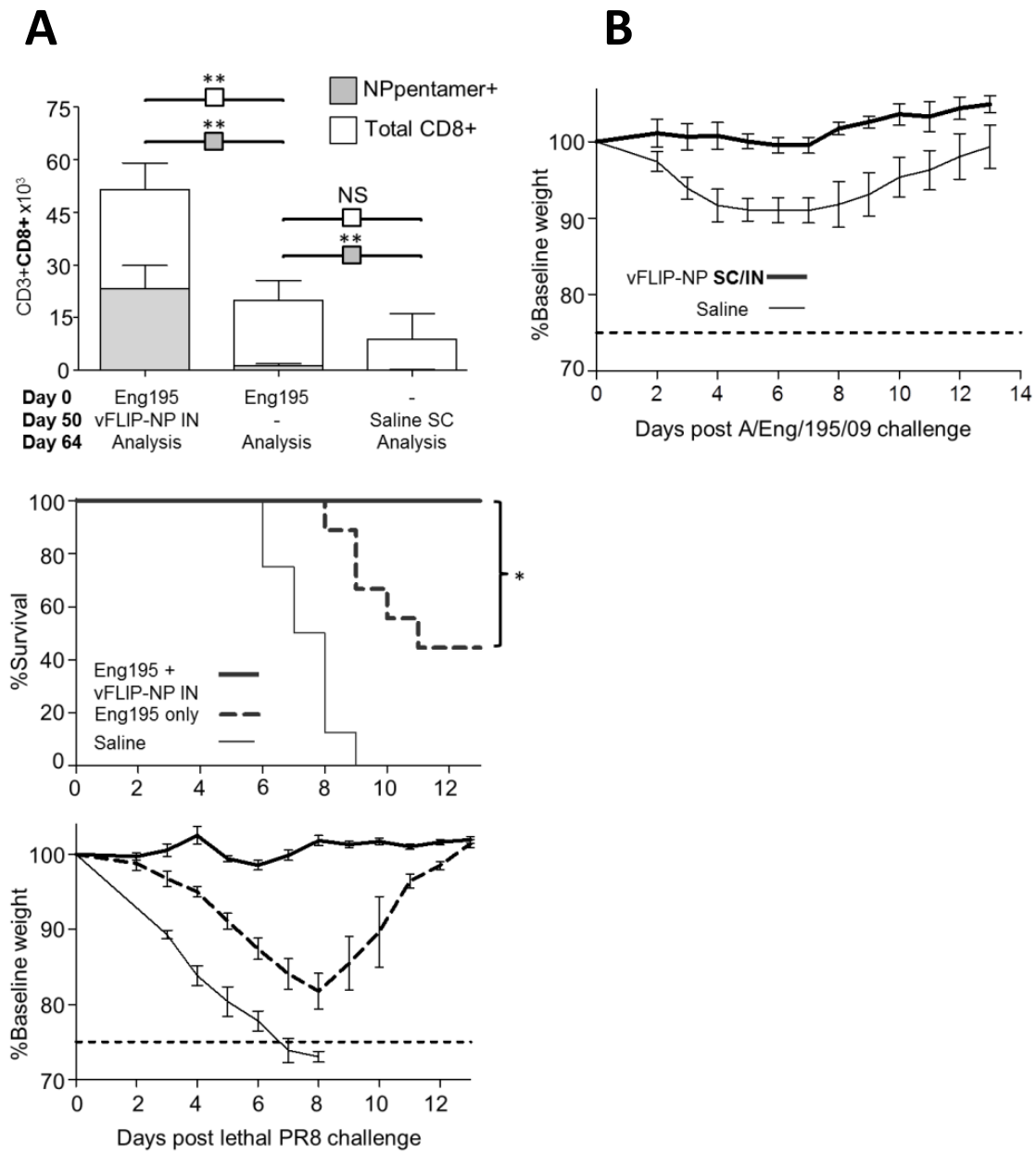


Figure 5-6 (A) Recall of naturally-acquired T cell memory to the lung by intranasal vFLIP-NP LV. Top: Lung total CD8 and superimposed $NP_{147-155}$ pentamer CD8+ T- 64 days after non-lethal challenge with A/Eng/195/09, with and without intranasal vFLIP-NP boosting 2 weeks before analysis (n=4 per group). Percentages of total CD8+ T cells that are $NP_{147-155}$ pentamer +ve are shown within bars. Survival and weight loss in survivors (n=12 per group) are shown beneath following challenge with lethal A/PR/8/24 64 days after prior non-lethal infection with A/Eng/195/09. Mantel-Cox tests and Gehan-Breslow-Wilcoxon Test produced similar p-values for observed differences in survival. **(B)** Cross-strain protection against A/Eng/195/09 conferred by SC-IN immunisation with LV vFLIP-NP. Mice (n=10 per group) were challenged with $5x10^4$ PFU of A/Eng/195/09 two weeks after the final immunisation and weights monitored as previously.

5.3.4 Nonintegrating LV prime systemic T cell responses but do not establish lung-based T cell populations by intranasal recall

vFLIP-NP LV were produced using a packaging plasmid encoding a defective integrase (vFLIP-NP NI). We and others have previously demonstrated this leads to an integration rate 10,000 fold lower than LV produced with a WT integrase[475,478]. Mice were vaccinated with a single dose of 200 ng RT vFLIP-NP NI SC and splenocytes analysed 2 weeks later for NP-pentamer+ CD8+ T cells or IFNγ responses upon re-stimulation with NP peptide. This revealed that vFLIP-NP NI generates a detectable CD8+ T cell response as measured by pentamer and IFNγ expression after re-stimulation. However, the responses were less than those seen with WT vFLIP-NP or Null-NP vaccination(Figure 5-7). SC-IN vaccination with vFLIP-NP NI failed to induce lung-based NP-specific T cells, despite giving a higher dose of 800 ng RT IN, although IN administration did appear to non-specifically increase the number of CD8+ T cells in BAL. This increase was approximately 7-fold, compared with a 60-fold increase induced by WT vFLIP-NP. Correspondingly, protection was not enhanced beyond the level seen with SC vaccination with WT vFLIP-NP.

5.3.5 Human monocyte derived DC transduced with LV can recall NP-specific autologous CD8+ T cell responses.

To test the principle that lentivirus-transduced APCs could recall human influenza T cell responses, we transduced monocyte-derived dendritic cells from HLA-A3 healthy volunteers (NP from A/PR/8/34 lacks a well-characterised HLA-A2 epitope) and co-cultured with autologous PBMC. These were then analysed for expansion of IFNγ or TNFα-secreting CD8+ T cells *in vitro*. 5 out of 7 HLA-A3 volunteers demonstrated a discernible expansion of IFNγ-secreting CD8+ T cells following expansion with peptide pulsed, IFNγ/LPS matured DC. This showed that monocyte-derived human DC transduced with vFLIP-NP LV stimulated significantly enhanced IFNγ and TNFα CD8+ T cell responses compared to Null-NP transduced DC, or DC matured with IFNγ and LPS and pulsed with the HLA-A3 restricted NP peptide. Use of LV expressing vFLIP with hepatitis B core (HBc) (or pulsing of LPS/IFNγ-matured DC with HBc peptide) did not induce an IFNγ T cell response, thus indicating these responses are NP-specific.

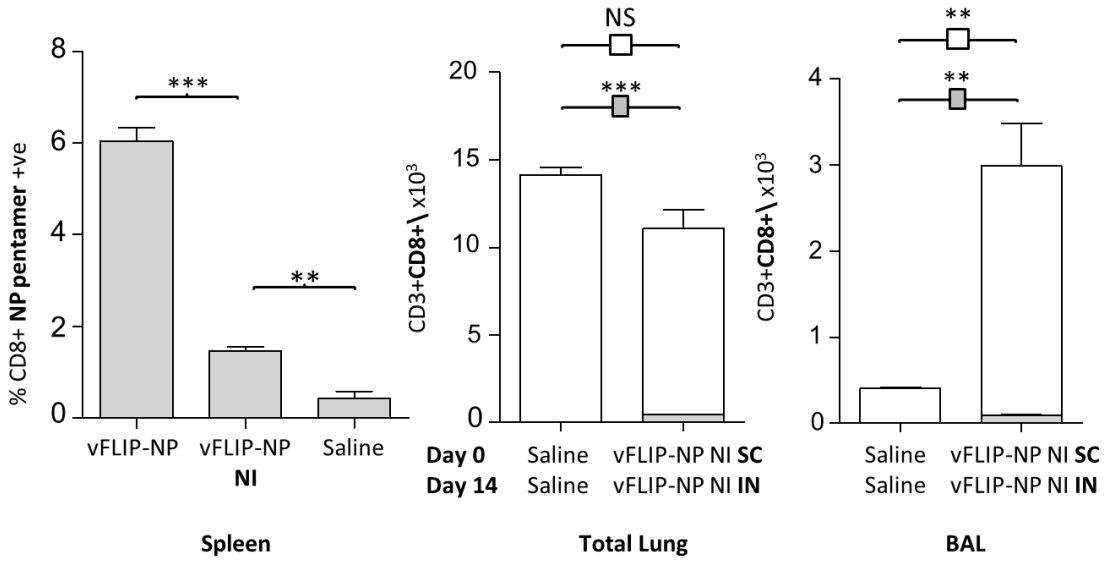


Figure 5-7 T cell responses in spleen, lung and airway after vaccination with non-integrating (NI) vFLIP-NP LV. Integrating vFLIP-NP responses are shown in splenocytes for comparison.

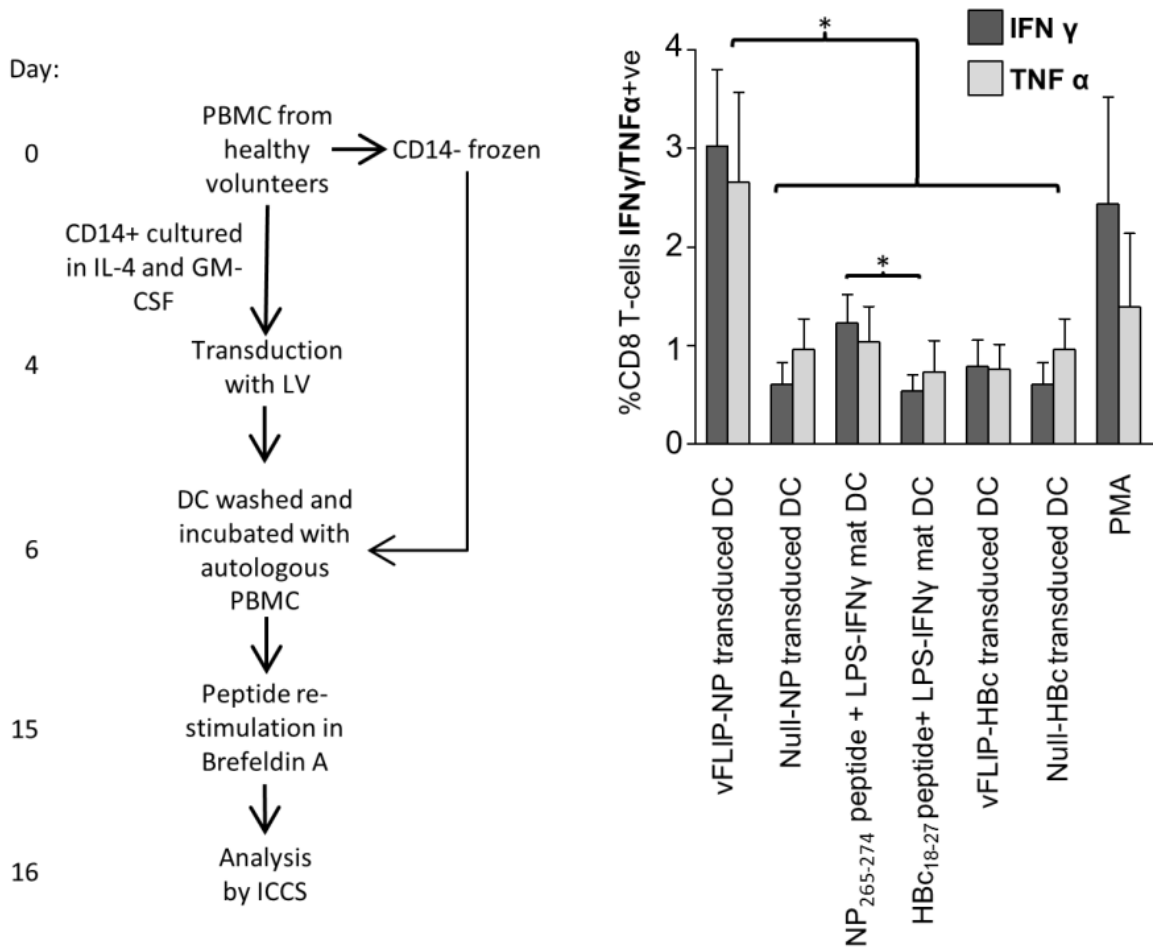


Figure 5-8 Human monocyte-derived DC transduced with LV expressing NP recall autologous T cell responses *in vitro*. PBMCs from HLA-A3 positive healthy volunteers were co-incubated with autologous DC that had been transduced with LV (or matured with LPS and IFNγ and pulsed with the HLA-A3 restricted peptide $NP_{265-274}$). Cultures were re-stimulated with $NP_{265-274}$ peptide prior to cytokine detection. To control for non-antigen specific stimulation DC transduced with LV encoding HBc were used. Data shown is for 5 out of 7 HLA-A3 subjects who responded to matured DC plus NP peptide. The % positive CD8+ T cells in each group were compared by the Wilcoxon matched pairs test.

5.4 Summary

The presence of NP-specific T cells in the lung and airway prior to challenge significantly shortens the duration of infection and peak viral titres compared with both naïve mice and those with systemic T cell immunity. The concurrence of high viral load and T cell number, rather than either in isolation, appears to drive cytokine production and lung injury. SC-IN vFLIP-NP vaccination leads to populations of lung-based NP-specific CD8+ T cells that are sustained for at least 4 months after the final vaccination. These confer 100% protection against lethal influenza challenge with minimal weight loss. A 4-month gap between SC and IN boost does not impair the size of the recall response to the lung. Mucosal T cell populations can also be boosted 60 days after primary infection with A/Eng/195/09 and confer complete protection against lethal A/PR/8/34, which is not neutralised by antibody mediated sterile immunity. SC-IN vFLIP-NP also protects against A/Eng/195/09 despite variation in NP sequence which includes the H2Kd-restricted CD4 epitope. SC vaccination with non-integrating vFLIP-NP induces NP-specific CD8+ T cells and confers partial protection against lethal A/PR/8/34 challenge but with clinical disease. SC-IN vaccination with vFLIP-NP NI does not induce mucosal NP-specific T cell populations in lung homogenate or airway. Human DC transduced with LV can recall *in vitro* autologous T cell responses against NP in HLA-A3 healthy volunteers. The strength of the recall response appears to be enhanced by vFLIP.

5.5 Discussion

Moskophidis *et al* were the first to interrogate the relative contribution of viral load and influenza-specific T cell number to histopathological change and morbidity/mortality in detail. They manipulated viral load (through influenza inoculation dose) in RAG-/- mice (lacking B cells and T cells) and $F5^{-}Rag^{-/-}$ mice expressing a uniform αβ TCR heterodimer mice such that 90% of CD8+ T cells are specific for an influenza A/NT/60/68 nucleoprotein epitope[482]. Unlike Rag-/- mice, F5

Rag-/- survived challenge at low challenge doses (<10^4 PFU A/NT/60/68). However, a key finding of this study was that F5-Rag-/- mice infected with a high inoculum (10^7 PFU) died significantly faster than Rag-/- challenged with the same dose. Histological analysis showed that there was less histopathlogical change in lung sections from Rag-/- mice than F5 Rag-/- mice at an equivalent level of viral load, and injury developed more rapidly in the latter. The addition of anti-IFNγ mAb during infection lessened histopathological damage but had no impact on survival. The authors concluded that *"the challenge in creating a CTL-based vaccine directed against heterosubtypic influenza virus strains is to raise the abundance of CTL precursor cells early in the infection in order to increase the protective response without exacerbating a pathology that is also CTL dependent*".

We have shown this is achievable through SC-IN vFLIP-NP vaccination, inducing a large lung-based CD8+ T cell population which secures rapid viral elimination and low peak titres such that stimulation of a deleterious secondary effector response is altogether avoided.

Further important inferences can be made from these data:

- Local, but not systemic, T cell responses are proportionate to viral load. The lower peak viral load seen in SC-IN vaccinated mice leads to lower peak lung T cell numbers but splenic memory NP-specific populations increase in line with those in SC vaccinated mice with 14-fold higher lung viral titres

- Relatively few lung-based NP-specific CD8+ T cells are required pre-challenge (compared with peak T cell responses seen during infection) to clear a lethal challenge without clinical disease.

- Viral titres in unvaccinated mice given a sublethal challenge reach a higher peak than SC vaccinated mice given a lethal dose, but a slower and less vigorous T cell response means peak and cumulative cytokine levels were lower in naïve mice. This suggests that the concurrence of high viral loads and T cell

numbers, rather than either in isolation, are necessary to elicit high cytokine levels and lung injury.

- The absence of lung injury with the near-absence of a TNFα, IFNγ, IL-4, IL-10, or IL-17 response corroborates the previously described role of these cytokines in deleterious histopathological change in influenza.

The shortened duration of infection and 14-fold lower peak viral loads seen in SC-IN versus SC vaccinated mice strongly supports the use of vaccination approaches that aim to maximise mucosal rather than systemic T cell immunity. The impact of this approach has yet to be modelled in the same way as the impact of systemic T cell memory on heterosubtypic immunity. As discussed in the introduction, extrapolations from models arrived at through analysis of lung CD8+ T cell number during primary and secondary responses predict that high numbers ($>10^5$) of lung-based T cells would be necessary to have any impact on peak viral load or clearance time[482]. This is because the rate at which infected cells are killed would have to be very high to limit replication if susceptibility of uninfected cells remains undiminished by the as yet minimal I IFN response in the first 2 days after infection. In absolute terms, SC-IN vFLIP-NP vaccination seems to achieve around $1.5\text{-}2x10^4$ NP-specific T cells, although we have used a non-enzymatic extraction technique and true numbers may be significantly higher. Nevertheless, this is sufficient to achieve a level of protection that prevents signs of clinical disease and is likely to considerably reduce the infectiousness of the vaccinated individual.

The duration for which this level of protection is maintained is unclear from these data, since it may extend beyond 4 months. Memory cells in the secondary lymphoid are stably maintained by IL-15- and IL-7-driven homeostatic proliferation [483–485]. However, the factors that influence numbers of memory T cells at peripheral sites of infection (or vaccination) are unknown. T cells primed by skin-derived DC express a skin-homing phenotype including E-selectin and P-selectin ligands and chemokine receptor 4 (CCR4) and CCR10[486–488]. Similar "imprinting" for intestinal migration results from priming by specialized DC subsets in the Peyer's patches or mesenteric lymph nodes[489,490], inducing CCR9 and integrin $\alpha_4\beta_7$ expression in activated T cells which

recirculate to the intestinal mucosal due to high selective expression of MAdCAM-1, the ligand for integrin $\alpha_4\beta_7$, on the surface of high endothelial venules and constitutive expression of C-C chemokine ligand 25 (CCL25), the ligand for CCR9, on intestinal epithelial cells. It is not known whether lung-draining DC imprint a particular migration pattern favouring the lung parenchyma or the airway, or whether antigen or chemokine signals are each sufficient to maintain recruitment from the circulation which appears to be the principal means by which these populations are maintained after infection[461,491492,]. Using a series of parabiotic mouse pairings, Zammit *et al* explored the requirement for antigen to maintain lung and airway influenza-specific CD8+ T cells after infection. When mice recently infected with X31 were conjoined with syngeneic naïve mice, NP-specific CD8+ T cells from the donor rapidly equilibrated with the naïve mouse in the spleen and circulation but these appeared much later in the lung parenchyma of naïve mice and none appeared in the airway. If both mice primed with X31 infection prior to being conjoined, T cells from each mouse (CD45.1 or CD45.2) would recirculate to the lung and airways of the other. To determine whether migration to the lung and airway was dependent on antigen or other inflammatory signals, mice primed intranasally with OVA-expressing listeria were conjoined with A/X31 infected mice. OVA-specific T cells could not be detected in the lung or airway of the latter, suggesting recruitment to this site is antigen-specific rather than dependent upon non-specific inflammatory signals. In a further experiment in this study, CFSE stained CD8+ T cells were transferred from F5 RAG-/- mice transgenic mice (described above) into mice infected 60 days previously with E61-13-H17 influenza (expressing the F5 TCR CD8 T cell epitope). Adoptively transferred T cells proliferated rapidly in the lung-draining lymph nodes but not in the lung parenchyma itself, indicating the presence of antigen in the former two months after infection. The absence of detectable influenza RNA at this time point suggests antigen is preserved without replicating virus, perhaps as immune-complexed antigen on the surface of follicular dendritic cells. How this lymph-node centred proliferation of influenza-specific T cells after infection maintains lung and airway T cells is unclear. Airway-based influenza specific CD8+ T cells appear to be recruited continually from the circulation with a rapid turnover (90% every 10 days). The precise signals governing this chemotaxis are unknown, although the expression of BTL-1 (the

receptor for leukotriene B4) and the $\alpha_1\beta_1$ integrin VLA-1 which mediates retention of effector T cells at peripheral sites of inflammation may both play a role[493,494].

In Section 4.3.4 we identified a number of T cell chemoattractants that are secreted by vFLIP activated AM. A simple of means of identifying the relative importance of each of these in generating and/or maintaining CD8+ T cell populations in the lung and airway would be to examine the relative expression of their respective receptors on airway-based CD8+ T cells after intranasal LV. This would permit development of a more selective LV intranasal recall boost expressing NP and only the chemokine(s) necessary for T cell maintenance. This would avoid NFκB activation of large numbers of AM, which risks disrupting local immunoregulation and inappropriately heighten responses to innocuous inhaled antigen.

We have not yet tested the longevity of lung-based T cell populations after boosting with Null-NP or vFLIP-GFP, which would determine the relative importance of antigen versus chemokine secretion in the recall signal for mucosal memory maintenance. The longevity of LV-transduced AM in other studies (which we have not yet examined in our model) hints at the possibility of maintaining antigen presentation in the airway and chemokine secretion indefinitely. Whether this is desirable or not is an important question, since prolonged antigen exposure may risk T cell exhaustion, or the presence of long term T cells with low levels of activation may have unforeseen deleterious consequences in the lungs such as stimulation of fibrosis.

The ability to "switch off" antigen presentation or chemokine secretion by AM would afford on opportunity to investigate the requirements for maintenance of mucosal T cell immunity. Since clodronate liposomes (or intranasal diphtheria toxin in CD11c-DTR mice) eliminate all macrophages this approach kills untransduced AM and negates their immunoregulatory function. As discussed in Section 4.5, we have therefore developed a trivalent LV expressing antigen together with the diphtheria toxin receptor (either side of an internal ribosomal entry site (IRES)) driven by a PGK promoter, with vFLIP expressed from the SFFV promoter. Initial data suggests that AM transduced by an LV expressing GFP and DTR are completely eliminated by intranasal diphtheria toxin administered 4 days after LV, with preservation of large numbers of live untransduced AM. This approach may therefore provide a useful model by which

the impact of removal of transduced AM upon longevity of mucosal T cell populations can be studied.

The ability of T cells specific to NP to confer heterosubtypic protection is well established in mice. This is typically demonstrated using A/PR/8/34 (H1N1) and X31 (H3N2) which share identical nucleoprotein sequences. Variations in the immunodominant class I restricted epitopes between primary and secondary influenza infections are known to significantly impair heterosubtypic immunity in mice[495]. Fortunately there is a preponderance of influenza epitopes restricted by highly prevalent HLA subtypes which are both highly conserved and immunodominant. However, the degree to which variation in other less conserved epitopes impacts on cross-protection is less clear. Such variation may disrupt class II restricted epitopes and therefore impair CD4+ T cell secondary responses, or generate class I restricted epitopes that compete with the immunodominant epitope for processing and presentation. The emergence of A/Eng/195/09 during this work provided an ideal test of the ability of T cells generated by SC-IN vFLIP-NP vaccination to protect against a strain with only 91% NP amino acid homology (but preserved a preserved H2Kd restricted epitope). We have shown that A/Eng/195/09 infection does generate some degree of protection against A/PR/8/34, although all mice lose weight suggesting this is not mediated by sterilising antibodies against HA or NA. This immunity can be substantially boosted by intranasal vFLIP-NP to confer compete protection against A/PR/8/34 without weight loss. Furthermore, SC-IN vaccination with vFLIP-NP confers complete protection against A/Eng/195/09. Thus the absence of corresponding CD4+ T cell epitopes in the NP of A/PR/8/34 (and the LV vaccine) and A/Eng/195/09 does not appear to impair protection.

The lower transcriptional activity of non-integrating LV compared with integrated vector has been established both *in vivo* and *in vitro*. All previous reports of the use of NI LV for vaccination have required higher doses (often 10-fold or more) of the vector compared with WT LV in order to induce equivalent T cell responses[158,496,497]. Since we delivered 200ng RT in 40 µL to each mouse, this required a minimum concentration of 5 ng/µL which is at the upper limits of LV concentration using standard production protocols. Increasing this 10-fold is challenging (we have managed a maximum

concentration of 20 ng/µL). Scaling up production and increasing concentration for proof of principle in mice may be possible, but LV production for human clinical use would require a prohibitive magnitude of up-scaling with current production techniques. Furthermore, increasing the dose of NI LV may not necessarily correspond to increased efficacy, since there may be direct silencing of episomal DNA in murine AM. Further examination and optimisation of the level of expression of episomal transgenes in murine AM and DC is necessary.

The enhanced *in vitro* recall of NP-specific autologous human T cell responses by monocyte derived DC transduced with vFLIP suggests that NFκB activation of APCs may enhance T cell function at recall as well as at priming. Memory T cell recall responses are conventionally regarded as independent of APC-delivered co-stimulation[498,499]. However, in some models of secondary acute viral infection in mice, such as murine gamma herpes virus 68 (MHV-68) and vaccinia virus, CD28 stimulation has been shown to be necessary for efficient CD8+ T cell recall[500]. In influenza, $CD28^{-/-}$ mice show impaired priming of influenza-specific CD8+ T cell responses and subsequent recall, although it is unclear to what degree the poor secondary expansion is a consequence of a failure to generate sufficiently responsive memory population at priming rather than at recall[501].

It is difficult to discount the possibility that the enhanced CD8+ T cell recall response seen with NFκB activation of DC is due entirely to increased MHC I expression and thus greater exposure of memory T cells to antigen. It would be useful to repeat this experiment using *ex-vivo* human AMs from BAL although this would be subject to the confounding factor of AM activation upon extraction from surfactant and the other immunoregulatory signals of the airway environment discussed above. AM can be transduced *in vivo* by aerosolisation of viral vectors in primate models[502], although the majority of clinical gene therapy trials for cystic fibrosis have hitherto only attempted to demonstrate transduction of nasal epithelium in which transduction is more readily verified[503].

In summary, vFLIP-NP SC-IN vaccination shows promise across a range of scenarios analogous to its application in the clinical domain. However, in addition to the safety

concerns regarding integrating LV there are major hurdles to overcome for the expanded use of lentiviral vectors in Phase I/II clinical trials. This requires efficient, large-scale production under current Good Manufacturing Practice guidelines. A number of investigators, including this group, have addressed this by creating packaging cell lines to produce LV[504–507]. These can be grown in suspension in large bioreactors to generate high volumes of LV. Many of these have used inducible expression systems to avoid the cellular toxicity from HIV proteins and the VSV-G envelope. However, a recurring issue is vector gene silencing over time with increasing passage, resulting in reduced titres[508]. Furthermore, packaging cell lines still require stable transfection with the chosen expression vector and re-selection of clones for production which is time-consuming. Therefore whilst small scale clinical trials are feasible (and on-going), significant improvement of LV production would be required before they could be used cost-effectively in mass vaccination programmes.

Thus, whilst the level and duration of protection achieved with LV SC-IN in mice is compelling, the value of this vaccination modality probably lies for now in its advantages as an investigative tool, in particular the ability to discern the immunogenic function of an encoded adjuvant from minimal vector-related responses and also to target a wide range of immune cells by different routes of administration and thus determine their role in immune responses. In the following chapter we exploit these characteristics to evaluate the potential of vectored 4-1BB ligand to enhance responses against co-encoded antigen.

6 4-1BBL as a vectored vaccine adjuvant

6.1 Introduction

6.1.1 APC activation versus selective co-stimulation

Optimising T cell responses for prophylactic or therapeutic applications has for the most part focused on generating high quantities of functional memory or effector T cells, with a focus on their cytotoxic and cytokine-secreting capability. In previous chapters we have shown how vFLIP mediated specific NFκB activation of DC and AM leads to an activation phenotype that favours efficient T cell priming and recall respectively. Importantly, this generates high quantities of T cells which appear effective at clearing influenza-infected targets.

However, sustained NFκB activation of AM (and epithelial cells) following intranasal administration of vFLIP-expressing vectors may be undesirable for several reasons. vFLIP has been shown to have oncogenic potential, as evidenced by an increased incidence of lymphoma in vFLIP transgenic mice. This appears to occur through constitutive up-regulation of NFκB and consequent increased sensitivity to mitogenic stimuli, such as c-myc[509], rather than through directly inhibiting apoptosis, since Fas-dependent apoptosis pathways are preserved[510]. There are alternatives to vFLIP for NFκB activation, such as vectored over-expression of an NFKB inducing kinase (NIK) which has similarly been shown to enhance DC activation and T cell responses against co-encoded antigen[511]. However, this does not circumvent the potential problem of non-specific activation other pro-inflammatory cells by indefinitely activated DC, which may precipitate autoimmune disease.

A safer, more targeted approach may be to transduce antigen presenting cells with antigen and a selected single co-stimulatory signal that is known to induce desirable characteristics in the T cell response. Lentiviral vectors are ideal for this purpose since their intrinsic low immunogenicity means that background vector-induced APC activation is minimised, isolating the effect attributable to the encoded co-stimulatory

signal. Supra-physiological up-regulation of a single co-stimulatory molecule has the potential to generate pathogen-tailored T cell responses in which phenotypes that clear infection are maximized and those that are redundant or contribute to inflammation and injury are minimized. The use of endogenous co-stimulatory molecules also has the benefit of avoiding the often pleiotropic and unpredictable effects of viral adjuvants and their associated safety concerns.

In this chapter we explore the potential of a lentiviral vaccine expressing influenza nucleoprotein with the co-stimulatory molecule 4-1BB ligand (4-1BBL) which interacts with 4-1BB (CD137), a member of the tumour necrosis factor receptor (TNFR) family. The stimulation of 4-1BB on activated T cells by 4-1BBL expression on antigen presenting cells is increasingly recognised as an essential mechanism by which the longevity, size and functionality of T cell memory populations can be enhanced. Several aspects of this interaction make 4-1BB stimulation an attractive target for vaccination as discussed below.

6.1.2 TNF-receptor family signalling in T cells

The engagement of CD28 by its ligands CD80 or CD86 lowers the threshold for T cell activation and promotes IL-2 production in naïve T cells[512]. This is spatiotemporally associated with the interaction between the TCR and MHC-peptide complex and is thus termed “co-stimulation”. Following commitment to programmed expansion after recognition of antigen-MHC on an APC and CD80/86-CD28 co-stimulation, T cells continue to receive activation or survival signals throughout subsequent effector and memory stages.

The TNF receptor family have emerged as essential mediators of survival signaling in T cell subsequent to initial activation by CD80/86-CD28 interaction. They are expressed on both innate and adaptive immune cells and correspondingly can influence T cell responses both directly and indirectly. The principle members of the TNFR/TNF ligand family that have a direct effect on the T cell response are shown in Figure 6-1. These include OX40/OX40L, CD27/CD70, 4-1BB/4-1BBL, HVEM/LIGHT, GITR/GITRL and CD30/CD30L.

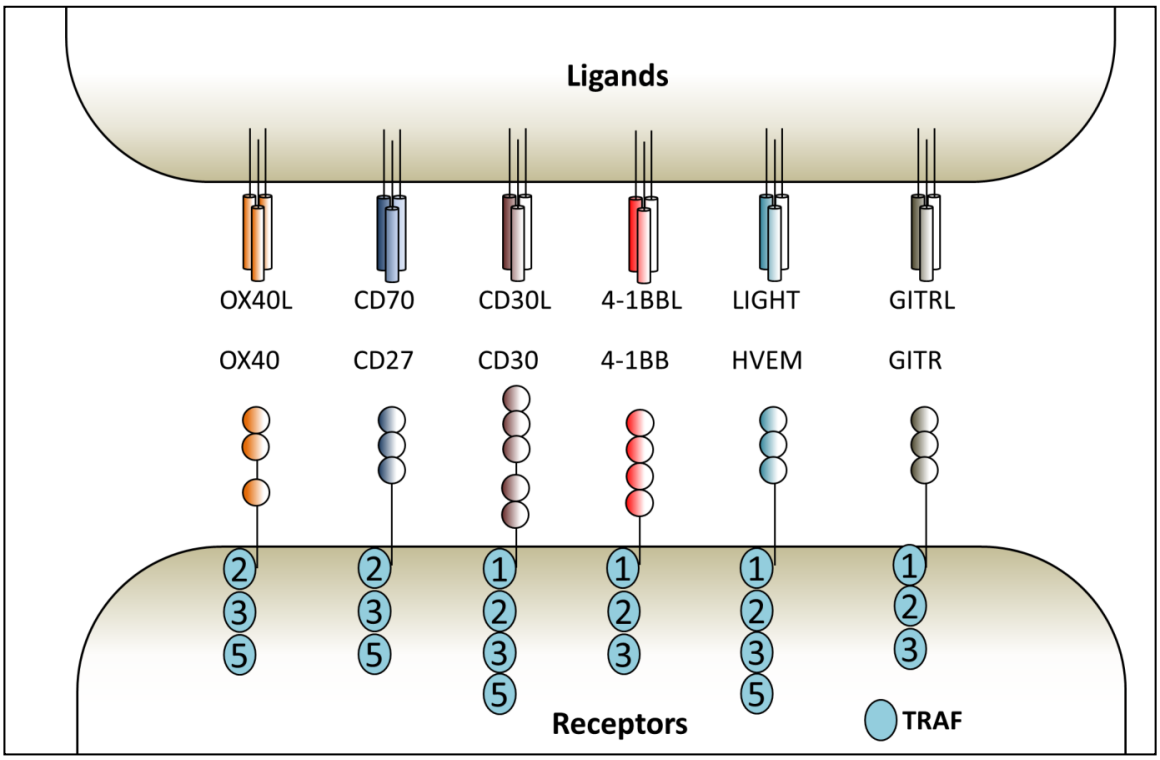


Figure 6-1 TNFR family and ligands. The binding of TNF-receptor associated factors (TRAFs) to each receptor is shown. All TNFR are capable of binding TRAF3 which inhibits signalling.

TNFR receptors can be sub classified into death-domain (DD)-containing receptors, decoy receptors and TNF receptor-associated factor (TRAF) binding receptors[513]. The first of these activate caspase cascades inducing apoptosis and include FAS, TNFRI and DR3. All TNFR involved in T cell signalling are able to recruit TRAF2 to their signalling complexes together with other TRAF proteins to varying extents, of which six have been identified so far[514]. The pattern of TRAF protein recruitment by different TNFR determines which downstream signalling pathways are activated and the consequential effects on T cell survival and functional differentiation.

Much of the importance of TRAF proteins in T cell signaling has been elucidated through their modification by truncation of RING finger domains which renders them dominant negative (DN) proteins. T cells with DN-TRAF2 fail to activate p38 in response

to 4-1BB stimulation[515] and have reduced survival following OX40 stimulation[516]. Other TRAF proteins seem less crucial to the central functions of T cell proliferation and survival. TRAF $5^{-/-}$ T cells, for example, exhibit normal IL-2 secretion and proliferation but produce higher levels of TH-2 cytokines in response to OX40 or CD27 signaling[517,518]. The principle TRAF proteins recruited by TRAF-binding TNFRs, their principle downstream signalling pathways and the functional consequences of stimulation in T cells are summarised in Table 6-1 below.

6.1.3 4-1BB signaling on T cells

4-1BB recruits both TRAF 2 and TRAF 1 after trimeric interactions between ligand and receptor. TRAF2 mediates stimulation of downstream signalling pathways NFκB (classical pathway) [519,520], JNK/SAPK[521] and p38 MAPK[522], whilst TRAF1 appears to enhance this interaction by reducing TRAF2 degradation[523]. T cell survival following 4-1BB stimulation is promoted by both ERK-dependent ubiquitination of the pro-apoptotic molecule BIM and by NFκB mediated expression of pro-survival members of the BCL-2 family, BFl-1 and Bcl-X_L[524]. Stimulation of cytokine production by 4-1BB signalling is mediated predominantly through TRAF2 dependent p38 activation[515] such that JNK and p38 activation can occur independently of TCR signalling during 4-1BB stimulation. However, a TCR signal is required for 4-1BB signalling to induce IL-2 production. This suggests there may be interaction between kinase pathways downstream of TRAF2 and the TCR although this has yet to be elucidated. 4-1BB may also reciprocally influence TCR signalling; cross-linking of 4-1BB has been shown to induce tyrosine phosphorylation of TCR-signalling molecules CD3ε, CD3ζ, Lck and SLP-76 (SH2 domain containing leukocyte phosphorylation of 76 kDa) and co-localised these with TCR-MHC/antigen interactions in the immunosyapse[525].

Table 6-1. Summary of TNFR family distribution, expression, TRAF recruitment, signaling pathways and function in T cells

Receptor/ Ligand	Receptor expression	Ligand Expression	Timing of expression of receptor in T cells	TRAF receptor recruitment and downstream signaling	Function in T cells
OX40/OX40L	T cell restricted. (CD4>CD8)[526] TH2>TH1[527]	Activated B-cells[528] Avtivated T cells[529] CD40-ligand-activated DC[530] Endothelial cells[531]	48 hrs post T cell activation[532]	TRAF 2,3,5 NFKB activation[533] Sustains the expression of anti-apoptotic BCl-2 family members following CD28 signaling in a P-I-3K dependent manner[534].	Augments primary expansion and survival of CD4 memory T cells[535]. Enhances CD4 cytokine production[532]. Expands both CD8 and CD4 cells[536].
CD27/CD70	NK cells, B-cells Naïve CD4 and CD8 T cells[537]	Activated T cells, B-cells and DC[538]	Decreases post-activation[539]	TRAF 2, 5 and Siva-1 Alternative and canonical activation of NFKB[540] JNK activation[541]	IL-2 dependent enhancement of CD4 and CD8 T cell proliferation and cytokine production[542].
HVEM/LIGHT	Resting T cells, monocytes, immature DC[543]	Activated T cells[544]	Reciprocal expression of HVEM/LIGHT following T cell activation[545]	TRAF 1,2,3,5 CD28-independent NFkB activation[546]	T cell cytokine production and proliferation soon after T cell activation[547].
CD30/CD30L	Activated T cells, B-cells,NK cells, eosinophils[548]	Resting B-cells and activated T cells[549,550]		TRAF 1,2,3,5 Alternative and canonical activation of NFKB[551] P38 MAPK activation[552]	Enhancement of T cell proliferation and cytokine production (including TCR-stimulation independent IL-13 production)[553].

GITRL/ GITR	Activated T cells[554] CD4+CD25+ T-regulatory cells[555]	Endothelial cells B-cells, macrophages, BM-derived DC[556]	Peaks at 24hours post-activation of T cells[557]	TRAF 2 & 3 Siva-1 Apoptosis through Siva-1 signaling[558] NFKB activation through TRAF 2[559]	Stimulates proliferation of T cells in the absence of CD28 or TCR stimulation[560] Enhances T cell cytokine secretion[561]. Enhances proliferation and survival of T-regulatory cells[562].

4-1BB expression in T cells is itself dependent upon NFκB activation following TCR engagement with antigen (with the exception of consitutive expression seen on CD4+CD25+ regulatory T cells)[563]. Therefore, 4-1BB expression on T cells may be regarded as a mechanism whereby antigen-mediated activation NFκB activation can be sustained in an antigen-independent manner in the presence of 4-1BBL stimulation.

6.1.4 Regulation of 4-1BB expression and signalling in T cells and other tissues

4-1BB expression is not restricted to T cells and has been noted on B cells, monocytes, macrophages, dendritic cells and eosinophils[564–567]. It has also been demonstrated in non-immune tissues such as endothelial cells, smooth muscles cells, neurons, microglia and astrocytes[568–570]. Tissue-specific regulation of 4-1BB expression may occur through production of transcript variants under the control of different promoter regions and also by alternative splicing. In the mid-1990s, Kwon *et al* identified two different species of 4-1BB cDNA with varying 5' untranslated regions (UTR), raising the possibility that these are transcribed from different promoters[571]. Further analysis of the tissue-specific expression of these transcripts in the mouse revealed that the type I transcript is restricted to immune cells, whereas the type II transcript is ubiquitously expressed (and is also found in activated T cells). Three putative promoter regions were recently identified, of which promoter region 1 contains NFκB-binding elements and all of which contain AP-1 binding elements. The type I transcript (driven from promoter region 1) was increased following NFκB stimulation (induced by anti-CD3 stimulation) and mutation of either NFκB or AP-1 elements prevented anti-CD3 mediated activation of 4-1BB expression in T cells, with mutation of the NFκB element resulting in the greatest abrogation by far. Whilst this confirms previous observation of NFκB and AP-1 dependent expression of 4-1BB in T cells[572], it does not account for the lack of expression of 4-1BB in all tissues despite ubiquitous detection of a second transcript variant that differs from transcript 1 in the 5'UTR only. It can be postulated that this variable region confers susceptibility to an as yet unknown post-transcriptional regulatory factor although the function of such a regulatory mechanism is unclear. 4-1BB knockout mice develop normally, hence it seems unlikely that 4-1BB expression is required during development and later silenced at a translational level.

Furthermore, NFκB activation– of which all cells are capable - would seem an inadequate mechanism to account for tissue-specific expression of the Type I transcript. This suggests additional regulatory mechanisms are involved.

Alternative splicing of the type I transcript variant may be one such mechanism. This produces a soluble variant of 4-1BB (lacking exon 8) in a manner akin to several other members of the TNFR family (CD27, CD30, GITR, and Fas). These soluble variants are widely regarded as having an antagonistic role to their membrane-bound counterparts[573,574]. In T cells the soluble variant is induced to a similar degree as the normal variant by T cell activation. Transient transfection of increasing amounts of plasmid encoding soluble splice variant 4-1BB negatively inhibit T cell activation by stimulation through 4-1BB in a dose-dependent manner. This appears to occur, at least in part, through reduction in expression of the normal membrane-bound 4-1BB in the presence of the soluble variant. The production of the splice variant during T cell activation may thus provide a brake on potential positive feedback between 4-1BB and TCR signalling which prevents T cell hyperactivation[575].

6.1.5 4-1BB signalling on dendritic cells and other antigen presenting cells

In 2002 Wilcox *et al* demonstrated that 4-1BB is expressed on both splenic and bone-marrow derived murine DC of a CD11b+ CD11c+ I-Ab^{HI} phenotype. Stimulation of 4-1BB on DC by co-culture with 4-1BBL-transfected P815 cells resulted in high levels of IL-6 in the supernatant and, in combination with LPS, IL-12 was also detectable. This effect was neutralized by anti-4-1BBL mAb and reproduced by stimulatory anti-4-1BB mAb. Administration of the latter *in vivo* to RAG-1 knockout mice improved the ability of splenic DC from these mice to stimulate proliferation of allogeneic T cells and OVA-specific OT-1 cells, suggesting DC activation does not occur indirectly via 4-1BB activation on T cells. However, this study found no up-regulation of standard co-stimulatory molecules (CD80, CD86, CD40, 4-1BBL, Ox40L) on the surface of 4-1BB stimulated DC. A similar result was obtained by Futagawa *et al* who again demonstrated increased IL-12 secretion by DC stimulated with 4-1BBL-transfected p815 cells, although in this instance CD80 and CD86 expression were also increased[567].

This group also developed a mAb (TKS-1) against 4-1BBL capable of blocking DC activation in this system. The downstream signalling pathways activated by 4-1BB in DC is presumed to be the same as those in T cells hence it is postulated that DC maturation occurs through a combination of NFκB, ERK and p38 MAPK stimulation via 4-1BB recruitment of TRAFs as described above. This complicates studies examining T cell responses to 4-1BB stimulation since this may occur both directly through T cell 4-1BB expression and indirectly through DC activation. A further complication is the observation that reverse signalling occurs through 4-1BBL when expressed on DC. Soluble 4-1BB receptor cross-linked by Fc or anti-4-1BBL mAb induces proliferation of both human monocytes or mouse bone-marrow macrophages whilst also stimulating IL-6, IL-8 and TNFα secretion[576,577]. Two additional studies have shown that DC maturation can be achieved solely through reverse 4-1BBL stimulation, finding that the autocrine maturation action of TNFα secretion is the most likely mechanism[578,579].

Reverse signalling is not unique to 4-1BBL amongst the TNF receptor/ligand family, but little is known about the mechanism. Both human and mouse 4-1BBL cytoplasmic tails share a consensus sequence (ser/thr-X-X-ser/Thr) for phosphorylation by casein kinase I (CKI) which is required by another TNFR family ligand, Fas-ligand (FasL) for signalling through nuclear factor of activated T cells (NFAT)[580]. An alternative mechanism by which 4-1BBL may activate DC is through membrane-level interaction with TLR4, which has been shown to interact with 4-1BBL in yeast two-hybrid screens. This interaction requires the transmembrane and cytoplasmic domain of 4-1BBL but not the extracellular portion. Consistent with these findings, 4-1BBL deficient macrophages cannot sustain TNFα production in response to LPS beyond the time-point at which LPS normally induces 4-1BBL expression in these cells (12-18 hours). A similar defect was not observed in 4-1BB deficiency[581]. Importantly, however, 4-1BBL has no impact on early TNFα induction via TLR mediated stimulation of NFκB, AP-1, ERK or JNK activation, suggesting 4-1BBL expression assists the prolongation of DC immunoactivation rather than its initiation, similar to the role of 4-1BB in T cell activation.

The complexities of 4-1BBL/4-1BB signalling do not end there. The fact that both receptor and ligand can be expressed on activated DC, and both can signal, suggests some reciprocal control of their expression must exist without which a positive

feedback loop of stimulation might be readily generated. Indeed, 4-1BB appears to strongly suppress expression of the ligand. This is demonstrated by the up-regulation of 4-1BBL in LPS-stimulated DC from 4-1BB$^{-/-}$ deficient mice to a far greater degree than is seen in WT [582]. The reverse does not appear to be true, since 4-1BBL$^{-/-}$ deficient mice show only minor increases in 4-1BB expression. It is as yet unknown whether the surface down-modulation of 4-1BBL by 4-1BB is achieved through their respective signalling mechanisms or direct protein interaction in the membrane or cytoplasm.

6.1.6 The role of 4-1BBL:4-1BB signalling in T cell responses

Early studies of the proliferative potentiation by 4-1BB stimulation of T cells found it comparable to anti-CD28 stimulation in mouse splenocytes *in vitro*, whilst also preventing apoptosis of daughter cells as measured by DNA fragmentation[583]. The relative potency of 4-1BB stimulation versus other forms of co-stimulation is highlighted in knock-out mouse models in which CD28 co-stimulation is absent. In these systems, 4-1BB co-stimulation alone can readily generate T cell division, survival and effector function[584]. Indeed as an adjuvant delivered in adenovirus-based vaccines, 4-1BBL can generate more fully differentiated CD8 T cells against EBV or influenza antigens than co-expression of CD80[585].

These effects appear to be most pronounced in CD8+ rather than CD4+ T cells. A population of IFNγ- secreting and cytotoxic CD8+ T cells appeared to be selectively maintained by 4-1BB stimulation leading to more rapid graft rejection in a murine graft versus host disease (GVHD) model[586]. Correspondingly, systemic administration of anti-4-1BB antibodies to mice expands CD8+ T cells to a greater degree than CD4+ T cells[587]. Natural T-regulatory CD4+ T cells are an important exception to this, since these constitutively express 4-1BB without antigen activation[588] and readily undergo 4-1BB-stimulated expansion[589]. 4-1BB stimulation of CD4+ T effector cells, however, simultaneously renders them refractory to suppression by T-regulatory cells, suggesting a mechanism whereby 4-1BB stimulation drives a more potent T cell effector response but at the same time promotes accumulation of a regulatory

population that will be on hand to rapidly suppress effector T cell function once 4-1BB stimulation ceases.

The selectivity of CD8+ T cell stimulation over CD4+ may be attributed to greater expression of the 4-1BB receptor on activated CD8+ compared with CD4+ T cells[590]. This is borne out in an influenza and lymphocytic choriomeningitis virus (LCMV) model of infection of 4-1BBL deficient mice, wherein 4-1BBL appears to be necessary only for CD8+ T cell memory generation. In human anti-HIV[591]and anti-influenza[585] T cell recall responses, 4-1BB co-stimulation enhanced expansion of CD8+ T cells with polyfunctional cytokine secretion and higher perforin and granzyme A staining upon re-stimulation *in vitro*. This was particularly striking in long-term HIV-infected individuals suggesting 4-1BB co-stimulation may rescue CD8+ T cells from their "exhausted" state during chronic infection, restoring functionality through TRAF mediated BIM downregulation[592]. This highlights an important unresolved question - whether 4-1BB stimulation actually modulates T cell effector function directly or instead promotes survival of the most differentiated effectors. In mouse influenza models, the presence of 4-1BB stimulation at priming appears to dictate only the number of antigen –specific CD8 + T cells rather than proportions of polyfunctional effector T cells[593]. Furthermore, in the chronic LCMV clone 13 infection model, 4-1BB expression on CD8+ T cells is persistently high yet these cells remain functionally impaired consistent with an exhausted phenotype.

The duration and severity of infection appear to be important factors in 4-1BB-mediated T cell stimulation since they determine both 4-1BB expression on T cells and expression 4-1BBL on APCs. Non-replicating antigens (such as ovalbumin) result in only transient 4-1BB expression on T cells whereas persistent antigen presentation, as seen in chronic or severe infection or allograft rejection, leads to prolonged up-regulation of 4-1BB[594]. In influenza models of BALB/c mice, A/PR/8/34 infection leads to sustained expression of 4-1BB on lung CD8+ T cells, whereas the milder, non-lethal X31 strain results in only transient 4-1BB expression. Correspondingly, 4-1BBL expression is only significantly up-regulated in lung monocytes in A/PR/8/34 influenza and not infection with the milder A/X/31 strain. The mechanisms governing increased 4-1BBL expression in APCs in severe or prolonged infection remain largely uninvestigated. However, it has been observed that 4-1BBL deficient mice infected with A/PR/8/34 accumulate fewer

CD8+ T cells in the lung correlating with higher peak lung viral titres and increased mortality[595].

4-1BB stimulation at T cell priming appears to modify not only the primary effector T cell response but also the secondary response upon antigen re-encounter. In a study by Hendriks *et al*, the relative contribution of three TNFRs – CD27, OX40 and 4-1BB - to T cell responses to influenza (strain A/NT/60/68) were compared by creating various crosses of recombinant mice lacking one or more of these receptors[596]. This revealed that both CD27 and 4-1BB made non-redundant contributions to the primary CD8+ T cell response to infection. In addition, however, an adequate CD8+ T cell memory expansion upon secondary challenge relied upon the presence of 4-1BB and OX-40 signalling during the primary infection. Since memory T cells are slow-cycling[597] and the capacity for enhanced secondary expansion in conserved for at least 6 weeks after priming in the context of 4-1BB stimulation, the authors speculate that this entails programming at the molecular level during the primary response.

In some circumstances, 4-1BB stimulation at priming appears dispensable for primary T cell effector expansion and activation. This may apply to situations in which there is strong TCR stimulation and co-stimulation through CD28 at priming, rendering the contribution from 4-1BB to the primary response redundant. For example, 4-1BBL has little impact on primary T cell responses to anti-CD3 stimulation[598] or in allograft rejection models[599]. In PR8 influenza infection, which is more severe and prolonged than in the A/NT/60/68 model used above, 4-1BBL is redundant for the primary response but remains essential for adequate secondary expansion upon antigen re-encounter[593]. Supporting this, 4-1BB signalling only seems to impact the primary response in situations where CD28 co-stimulation is limiting, such as in vaccination with lipopeptide without adjuvant[600], or infectious challenge of $CD28^{-/-}$ mice[601]. In the latter context, a single dose of anti-4-1BB antibody given during infection with influenza can correct the defective secondary response of T cells primed in the absence of CD28[602].

Even in the presence of strong CD28 co-stimulation during the primary T cell response, 4-1BB signalling appears to influence the capacity for secondary T cell expansion. This

seems to apply only to situations in which antigen is rapidly cleared, such as in either A/PR/8/34 or A/NT/60/68 infection. This can be modelled with a non-replicative antigen and powerful adjuvant, such as with OVA plus LPS vaccination in the OT-1 TCR-transgenic model, wherein 4-1BBL is dispensable for primary responses but necessary for recall reponses[603]. Conversely, where antigen is persistent in latent or chronic infection, such as γ-herpes virus MHV-68 infection, 4-1BBL-deficient mice have quantitatively equivalent antigen-specific T cell responses compared with wild-type. However, secondary effector CD8+ T cells in 4-1BBL-deficient mice display deficient degranulation and viral loads are consequently higher. Therefore whilst the presence or absence of 4-1BB signaling may not influence T cell number in the presence of persistent antigen, it may promote survival of cells with higher effector functions or directly modify cytotoxic T cell function[604]. This is consistent with the observation that anti-4-1BB antibody stimulation *ex vivo* can rescue function of "exhausted" T cells in chronic HIV infection, which occurs via modulation of the pro-apoptotic molecule BIM[605].

In most of these studies, the "programming" effects of 4-1BB signalling at priming appear to influence memory T cell capacity for expansion independently of the size of the memory pool generated by the primary response. However, tonic signalling through 4-1BB has also been shown to influence the size of memory T cell populations. Transfer of OVA-stimulated OT-1 TCR –transgenic T- cells into 4-1BBL-deficient mice results in a 2-3 fold decrease in adoptively transferred cells 3 weeks later compared to transfer into WT mice. This is despite a similar number of cell divisions in the transferred population over this time period, suggesting 4-1BB stimulation promotes survival rather than proliferation[606]. This raises the question of where 4-1BBL is expressed so that memory CD8+ T cells populations can be maintained in the absence of antigen. Given the enrichment of memory CD8+ T cells in the bone marrow, some have hypothesized that the 1-3% of CD34+ cells there that express 4-1BBL are the principal source of this tonic 4-1BB stimulation to memory T cells[607]. This lack of data in this area highlights how much more is known about the role, control, signalling and distribution of 4-1BB expression in immune cells than is known about the ligand.

In summary, 4-1BB signalling (Figure 6-2), appears to provide a “back-stop” to ensure an adequate primary response where CD28 co-stimulation is limiting and ensures memory T cell survival in the absence of prolonged antigen exposure. In chronic infections, 4-1BB signaling may influence T cell effector function rather than numbers, although whether this occurs via promotion of survival of more functional cells or by direct functional modification remains to be determined.

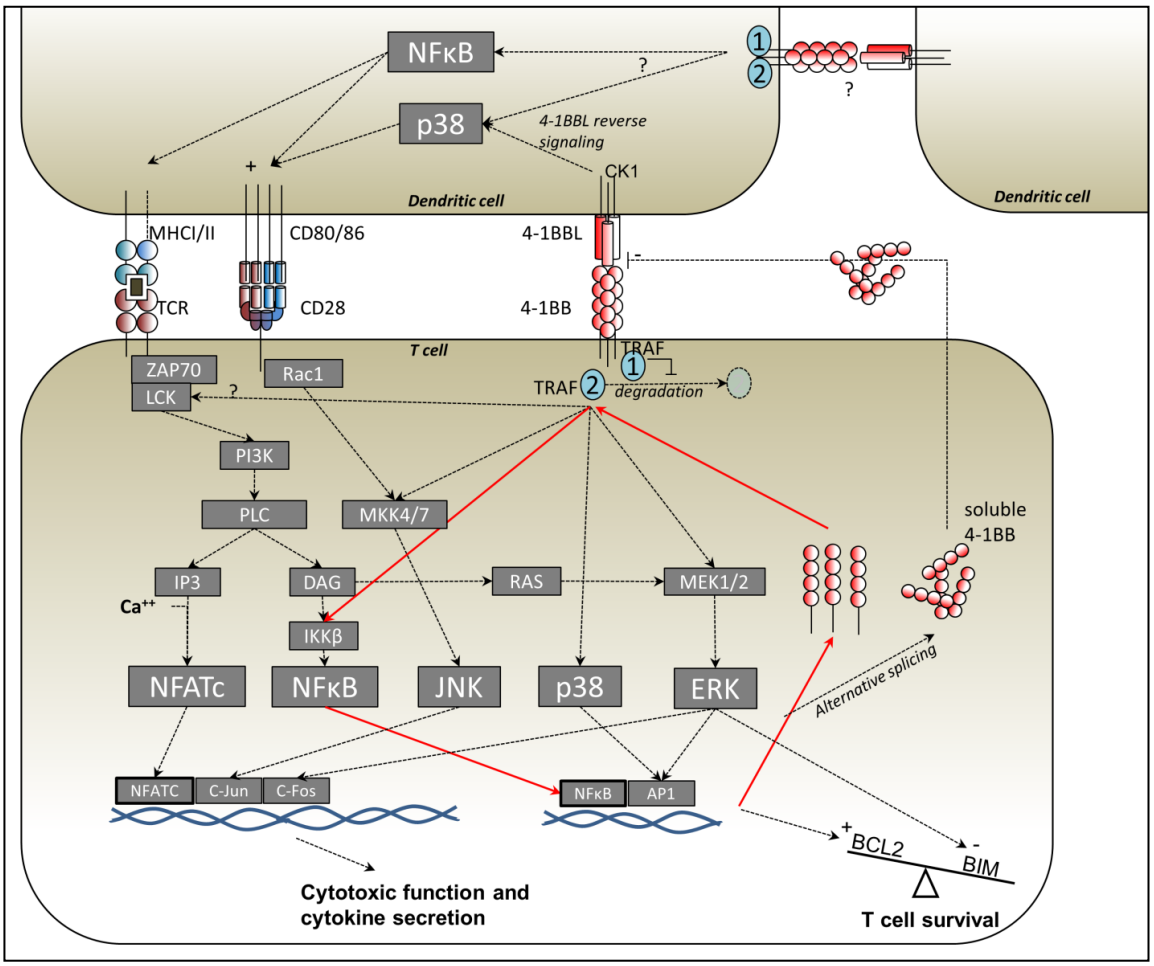


Figure 6-2 Summary of 4-1BBL-4-1BB signaling between DC and T cells. Red arrows indicates the dominant 4-1BB signaling pathway in T cells.

6.1.7 Current applications of 4-1BB stimulation for immunotherapy and vaccines

4-1BB stimulation thus modulates immune responses in a number of ways which may be suitable for enhancing responses to vaccination:

1. 4-1BB stimulation enhances T cell memory longevity and responsiveness to secondary stimulation
2. 4-1BB is expressed on activated but not naïve or resting memory T cells, hence stimulation through 4-1BB will enhance concurrent antigen-specific T cell activation but not non-specific T cell activation and autoimmunity.
3. The co-stimulatory signal is more potent for CD8+ T cells than CD4+, which may be useful in circumstances where a cytotoxic response is more desirable than pro-inflammatory T-helper cytokine responses.
4. 4-1BB stimulation may functionally enhance T cell responses, improving CD8+ T cell degranulation and preventing an "exhausted" T cell phenotype in prolonged infection.

Stimulation of 4-1BB has therefore been applied to improving T cell responses to infection and cancer, in both prophylactic and therapeutic contexts. This is achieved in a number of ways including anti-4-1BB mAb, use of soluble trimeric 4-1BBL and transgenesis of 4-1BBL either directly (using viral vectors or plasmids) or indirectly (via injection of 4-1BBL over-expressing cells).

4-1BB immunotherapy for cancer

Studies by Melero *et al* were the first to demonstrate stimulation of 4-1BB using 4-1BB specific mAb could promote effective anti-tumour T cell responses[608]. Further experiments by Miller *et al* showed that treatment with 4-1BB mAb was most effective soon after tumour challenge rather than before or during it[609], corroborating the observation that 4-1BB expression on T cells occurs more than 12 hours after activation *in vivo* and *in vitro*[610]. These improved anti-tumour responses appear to be CD8+ T cell dependent and are not abrogated in IL15-/- mice where NK cell

development is deficient or in MHC II-/- mice wherein CD4 responses are not elicicted[609].

An interesting observation in Miller *et al*'s study was synergy between treatment of tumour-bearing mice with 4-1BB mAb and flt3L, a cytokine that promotes proliferation of DC *in vivo*. This was attributed to the enhanced antigen presentation capacity increasing the number of CD8+ T cells on which 4-1BB could act, but since at the time 4-1BB expression on DC was not yet established, an alternative mechanism of 4-1BB stimulation of DC was not entertained.

Other investigators have transfected tumour cells with 4-1BBL and showed impaired growth together with induction of long-lasting tumour-specific T cells[611] or combined 4-1BB mAb stimulation with blockade of immunoinhibitory signalling through PD-1 to augment tumour responses (Xiao *et al*., 2007).

Translation of these findings to the clinic has been rapid, accelerated by the development of a well-tolerated panel of fully humanized anti-4-1BB mAb which was developed by Medarex Inc. One clone, 10C7, has been tested by Bristol-Myers Squibb in several Phase I and Phase II trials of solid tumour immunotherapy either as a single agent or with chemotherapy. Several of these trials, including a trial in unresectable melanoma patients, concluded in 2009 but results have yet to be formally reported.

4-1BB stimulation for vaccination against infection

Harnessing 4-1BB stimulation to improve prophylactic or therapeutic T cell responses against infection has not yet progressed to clinical trials but has been evaluated in a number of animal models. Among these, studies assessing the effect of 4-1BB stimulation on anti-HIV or -SIV responses are the most numerous. Harrison *et al* found that 4-1BBL, when included in the boost but not the prime of a vaccinia/fowl-pox prime-boost regimen, enhanced CD8+ T cell responses in mice against dominant HIV GAG and POL epitopes[612]. Ganguly *et al* similarly showed that expression of 4-1BBL in a DNA vaccine co-expressing GAG resulted in superior CD8+ T cell responses following MVA boost[613]. This study also investigated the expression of 4-1BBL in *cis* versus *trans*

with GAG and found the latter failed to enhanced CD8+ T cell responses above antigen alone, proposing that both antigen and 4-1BBL need to be expressed on the same co-transfected antigen presenting cell for optimal co-stimulation to antigen-specific T cells. A further interesting finding of this study was that anti-4-1BB mAb administered with DNA priming with GAG was a weaker adjuvant than co-expression of 4-1BBL with GAG, which achieved both greater CD8+ T cell and also antibody responses against GAG. Similar enhancement of CD4+ and CD8+ responses to DNA HIV-GAG vaccines was observed by Kanagavelu *et al* with the addition of DNA encoding a soluble form of 4-1BBL that is fused with surfactant protein D (SP-D), which forms a plus-sign-shaped molecule with four trimeric arms, thus multimerising 4-1BBL and ensuring cross-linking of target 4-1BB receptors[614].

The necessity for co-expression of 4-1BBL in *cis* with antigen in DNA vaccines was also not borne out in the study of Du *et al* which compared CD4+, CD8+ and humoral responses to an HBsAg DNA vaccine co-injected with a DNA plasmid expressing either 4-1BBL, OX40L or CD70 *in trans*[615]. 4-1BBL was consistently the most effective adjuvant in all three indices.

Much of the role of 4-1BB signaling in T cell responses has been established in mouse models of influenza. Accordingly, the effects of additional 4-1BB stimulation in T cell vaccination strategies against influenza have also been investigated. Moraes *et al* found that an intranasally administered adenovector expressing 4-1BBL and influenza NP generated superior lung CD8+ T cell responses and protection against PR8 challenge than an adenovector expressing NP alone. CD107a expression in antigen specific memory-T cells was also enhanced by inclusion of 4-1BBL in the adenovector. However, this difference was only demonstrable at low vaccination doses (10^4 PFU per mouse) leading the authors to postulate that the adjuvanticity of the immunogenic adenovector overwhelms any additional co-stimulation provided by 4-1BBL above this dose[616]. Furthermore, IM vaccination yielded no differences in T cell responses at any dose in the spleen or lung, which were only demonstrable following IN administration. In this study the authors also sought to discriminate the relative contribution to T cell expansion by direct 4-1BB stimulation of T cells and indirect stimulation via activation of non-T cells, such as 4-1BB expression on antigen presenting cells. To do this, bone marrow chimeric mice were established in which 4-1BB deficient mice were re-

constituted with a mixture of wild-type and $TCR^{-/-}$ bone marrow or a mixture of $4\text{-}1BB^{-/-}$ and $TCR^{-/-}$bone marrow. The former group have 4-1BB expression on all haematopetic cells whereas the latter only on non-αβ T cell haematopeotic cells. Mice were then vaccinated with adenovector expressing NP or NP and 4-1BBL and antigen-specific CD8+ T cell responses quantified by tetramer at day 10 in lung, MLN and spleen. Intriguingly, T cell responses in all compartments were significantly enhanced even in the absence of 4-1BB expression on T cells, although not to the same degree as in $WT/TCR^{-/-}$ mice in which 4-1BB expression was normal on both T cells and non-T cells.

In related work from the same group, 4-1BBL expressed with influenza NP on an adenovector has been shown to enhance human CD8+ T cell recall responses against influenza. Peripheral-blood derived monocytes were transduced with adenovectors expressing 4-1BBL (or empty) and pulsed with influenza (or EBV) peptides before incubation with autologous PBMCs and analysis of expansion and functional characteristics of peptide-specific T cells. This demonstrated greater expansion of peptide-specific T cells by vectors encoding 4-1BBL, together with up-regulation of granzyme-A, perforin and enhanced cytolytic activity at day 9[617].

6.1.8 A Lentiviral vaccine expressing 4-1BBL

K562 cells, which lack MHC expression and therefore do not induce allogenic T cell responses, have been stably transduced with lentiviral vectors expressing 4-1BBL to generate a stimulatory antigen-presenting cell line capable of long-term T cell expansion *in vitro* for immunotherapy purposes[618]. However, the potential of lentiviral vectors expressing TNFR family ligands as vaccines has not previously been explored. The ability of LV to transduce non-dividing antigen presenting cells and their relatively low intrinsic immunogenicity make these ideal vectors to explore the potency of 4-1BBL as a vectored adjuvant. In addition, the long-term expression achieved by integrating lentiviral vectors may have benefits for T cell memory longevity, since tonic 4-1BB stimulation has been suggested as a means by which memory populations are sustained independently of antigen. The high capacity of LV and ability to co-encode whole antigen together with 4-1BBL may ensure co-localised expression of both

antigen and co-stimulation, avoiding the pleiotropic and non-antigen specific immunostimulatory effects observed in mice after administration of anti-4-1BB antibody. Furthermore, since cross-linking by trimeric ligand is thought to provide the most potent stimulation through 4-1BB[619], presentation of ligand on the surface of transduced cells may provide a more potent 4-1BB signal than soluble ligand or anti-4-1BB antibody.

6.2 Aims

The aims of this study were:

1. To determine whether 4-1BBL expression together with influenza NP in a lentiviral vector (4-1BBL-NP) enhances T cell responses and/or protection against influenza in a mouse model of A/PR/8/34 infection compared with LV expressing NP alone.

2. To investigate indirect T cell stimulatory effects of vectored 4-1BBL, in particular via activation of DC.

6.3 Results

6.3.1 Lentiviral vectors encoding 4-1BBL transduce 293T cells and BM derived DC resulting in surface expression of 4-1BBL

Lentiviral vectors were cloned as described in the methods (page 93). Expression of 4-1BBL (mouse or human) was verified by surface staining of transduced 293T cells, BM-derived mouse dendritic cells, or human monocyte-derived DC on day 3 after transduction (Figure 6-3).

Endogenous 4-1BBL was not detected above isotype control staining on either mouse or human DC on day 4 of *in vitro* differentiation from bone marrow or peripheral monocyte precursors respectively. Transduction with mouse (m) or human (h) 4-1BBL resulted in substantial overexpression of 4-1BBL in mouse DC, 293T cells and human DC (Figure 6-3B). Dose-dependent expression of 4-1BBL was confirmed by addition of varying quantities of 4-1BBL-GFP to mouse DC (Figure 6-3C).

6.3.2 4-1BBL-NP enhances antigen-specific T cell responses

In order to determine whether vectored 4-1BBL enhanced T cell responses against co-encoded antigen, BALB/c mice were vaccinated subcutaneously with 4-1BBL-NP, Null-NP or saline and sacrificed 14 days later for splenocyte analysis (Figure 6-4).

CD4+ and CD8+ T cell IFN responses were assessed by ELIspot after overnight re-stimulation with class II or class I restricted peptide respectively as described in the methods. This revealed significantly greater CD8+ and CD4+ T cell responses to vaccination with 4-1BBL-NP than Null-NP in with both class I and class II peptide re-stimulation respectively. As in previous experiments with vFLIP-NP, CD8+ T cell IFNγ responses were more prominent than in CD4+ T cells. Splenocytes were also re-stimulated *in vitro* for 4 days with class II restricted peptide and supernatants analysed by cytometric bead array for a panel of 10 cytokines including IFNγ, TNFα, IL-4, IL-10, IL-17, GMCSF, IL-1α, IL-5 and IL-6. Significantly greater concentrations of TNFα, IFNγ

and GMCSF were found in supernatants in responses to class II restricted peptide re-stimulation in splenocytes cultures from 4-1BBL NP vaccinated mice compared with mice vaccinated with Null-NP. This resembles the findings of Li *et al* who demonstrated increased IFNγ and GMCSF secretion by T cells (and reduced TH-2 cytokine secretion) in tumour-draining lymph nodes following stimulation with anti-4-1BB antibody *in vivo*[620].

Intracellular cytokine staining of splenocytes after overnight re-stimulation with class I $NP_{147-155}$ peptide revealed a trend towards greater GzmB, IFNγ and TNFα expression in CD8+ T cells in the 4-1BBL-NP versus Null-NP vaccinated groups but this did not reach significance.

No significant difference in the percentage of $NP_{147-155}$ pentamer positive CD8+ T cells was seen in splenocytes from 4-1BBL-NP and Null-NP vaccinated mice, although there was a trend towards greater GzmB and Ki67 expression in antigen-specific CD8 T cells from the 4-1BBL vaccinated group, suggesting greater cytotoxic and proliferative potential.

6.3.3 4-1BBL vaccination enhances survival against lethal A/PR/8/34 challenge compared with mice vaccinated with Null-NP

14 days after subcutaneous LV vaccination mice were challenged with a lethal $2xLD_{50}$ dose of A/PR/8/34 and monitored for weight loss (Figure 6-5). As previously stipulated, mice were sacrificed if they lost more than 25% of their bodyweight. As in previous experiments with subcutaneous vaccination, all mice developed severe clinical syndrome of weight loss, tachypnoea, piloerection and hunched posture. However, around day 5, 6 out of 10 mice vaccinated with 4-1BBL-NP began to recover weight and returned to baseline at day 12, compared with only 2 out of 18 mice in the Null-NP group (p=0.003, Log Rank Mantel-Cox test). Thus 4-1BBL-NP vaccination conferred a significant survival benefit upon lethal influenza challenge compared with LV expressing NP alone.

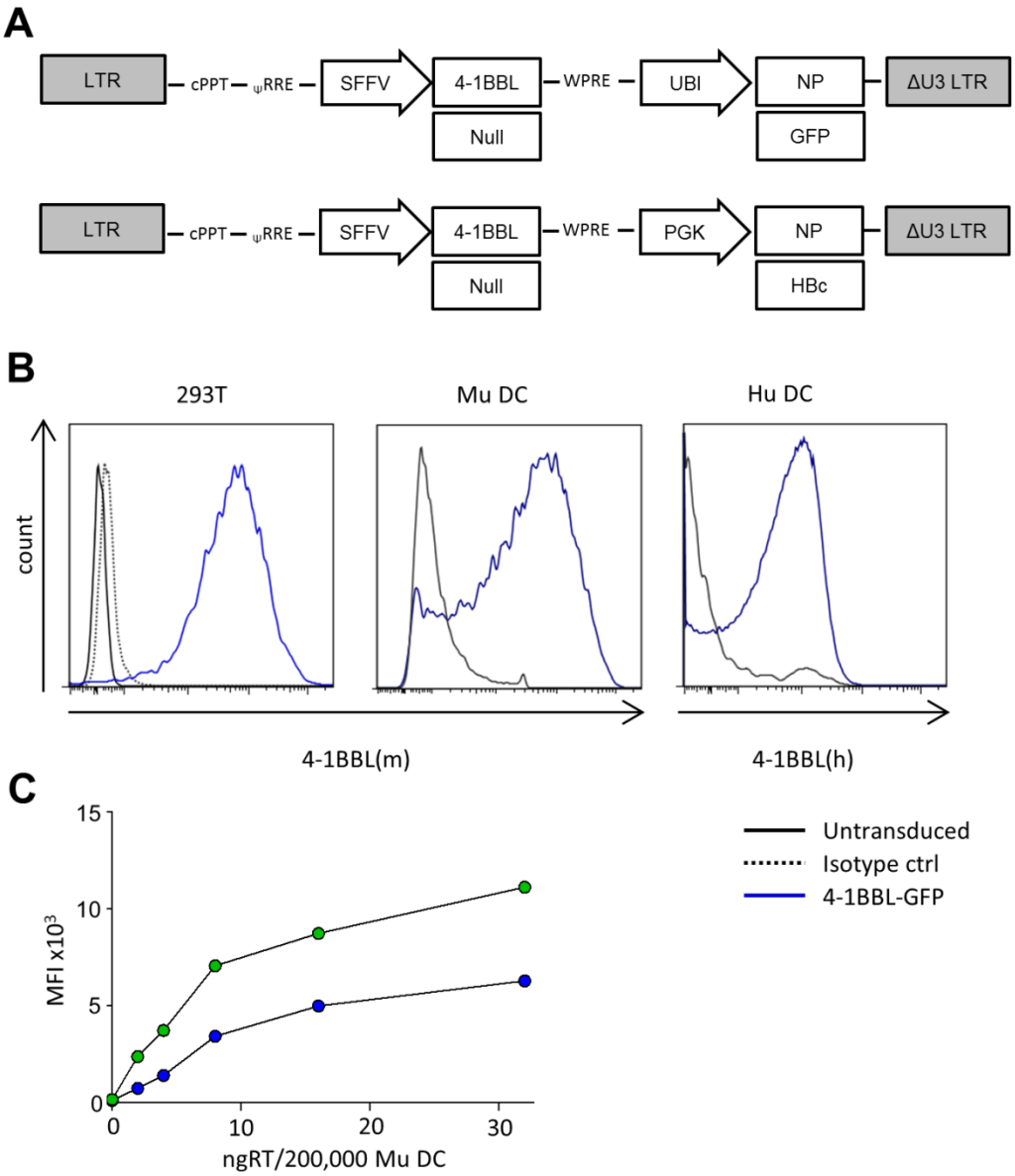


Figure 6-3 (A) Lentiviral vectors were cloned and produced as described in the methods. Constructs for mouse experiments used the ubiquitin (UBI) promoter at the second promoter, those for human experiments used the phosphoglycerokinase promoter (PGK) because the UBI promoter was found to be substantially weaker in human DC than murine. **(B)** Expression of mouse (4-1BBL(m) and human forms of 4-1BBL (4-1BBL(h)) in 293T cells, mouse bone marrow-derived DC (Mu DC) and human monocyte-derived DC (Hu DC). **(C)** Dose-dependent expression of 4-1BBL(m) (blue) and GFP (green) in mouse DC after transduction with 4-1BBL-GFP at varying doses.

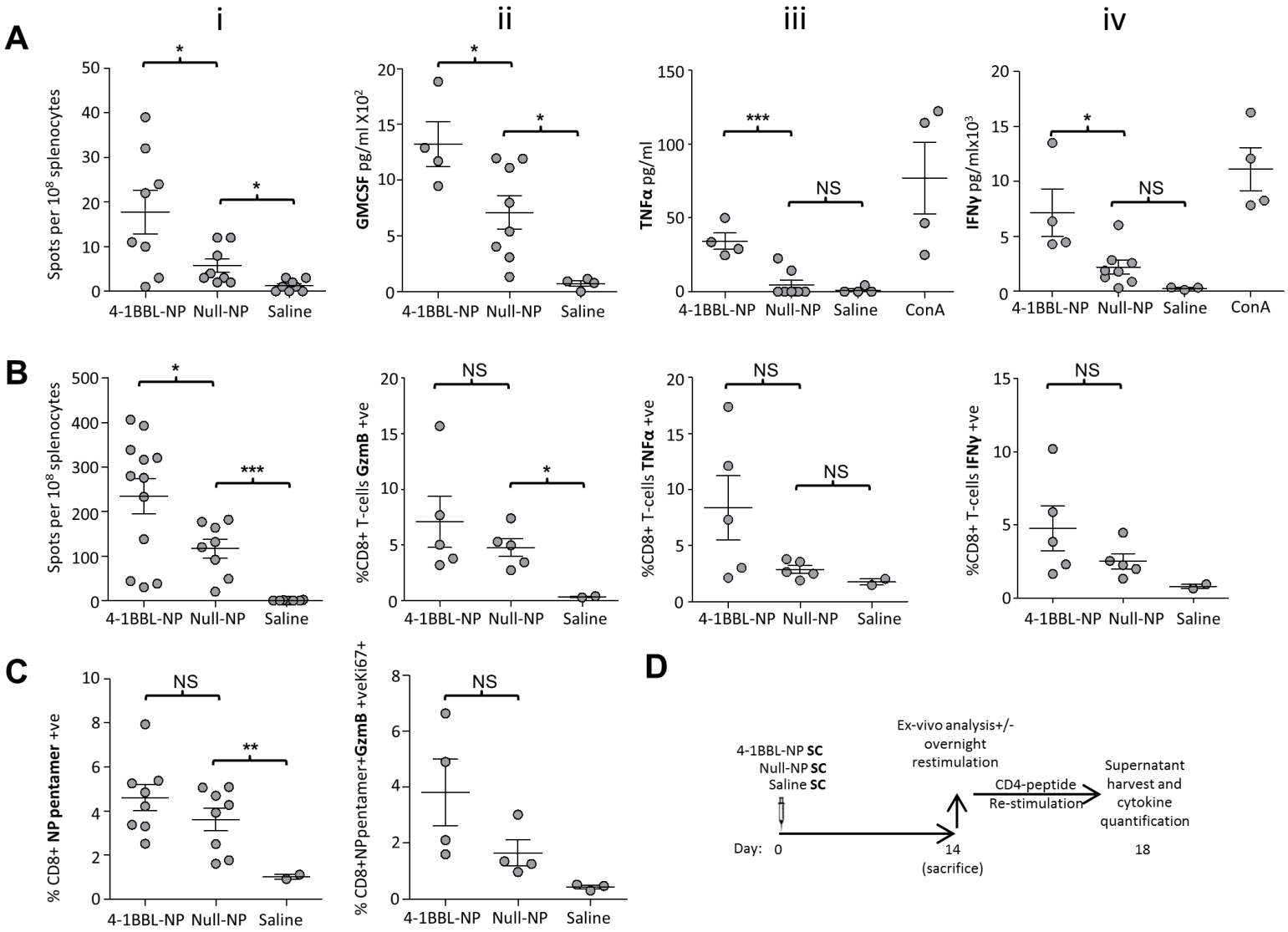


Figure 6-4 4-1BBL enhances mouse T cell responses against LV co-encoded NP. **(A)** CD4+ T cell responses after overnight re-stimulation with class II restricted NP peptide. (i) IFNγ ELIspot. (ii-iv) cytokine levels in the supernatants at day 4. **(B)** CD8+ T cell responses after (i) IFNγ ELIspot after overnight re-stimulation with class I restricted NP peptide. (ii-iv) Intracellular staining and FACS analysis. **(C)** CD8+ T cell NP pentamer responses (i) NP pentamer percentages of total CD8+ T cells in spleen (ii) percentage of GzmB and Ki67 positive NP-specific CD8+ T cells ex-vivo day 14 (no re-stimulation). **(D)** Vaccination and analysis schedule.

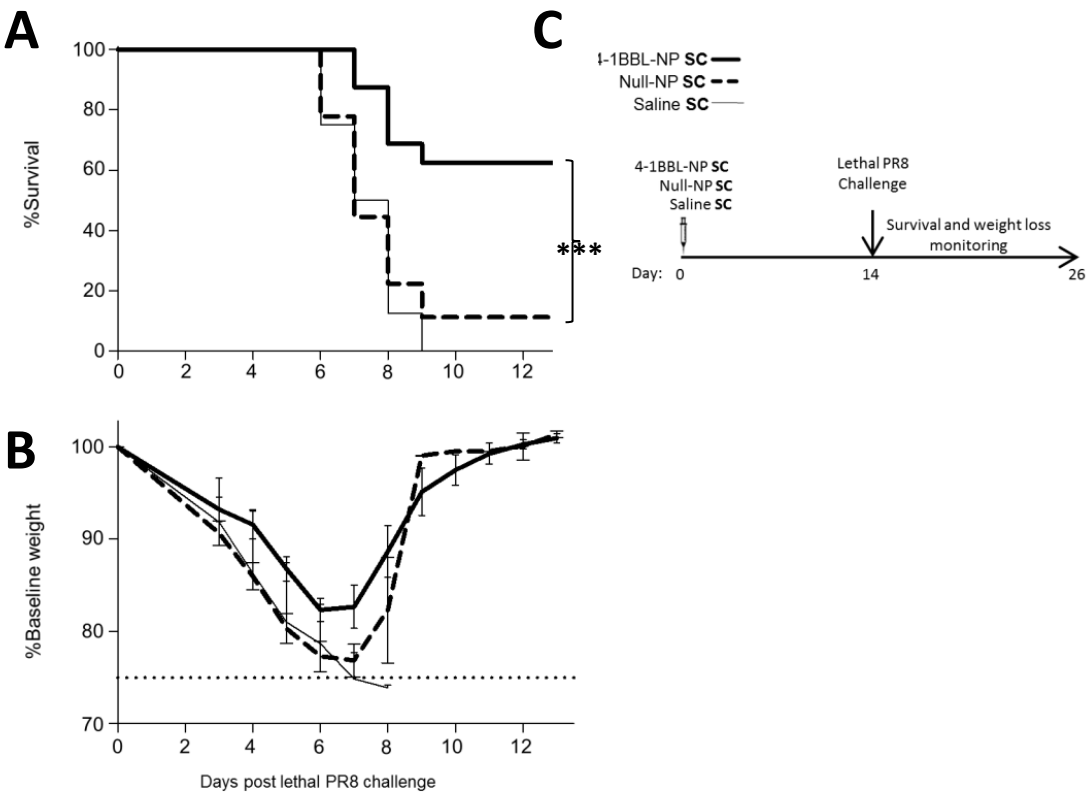


Figure 6-5 Survival **(A)** and weight loss **(B)** of BALB/c mice after subcutaneous vaccination and lethal influenza challenge according to schedule described in **(C)**. Both Mantel-Cox tests and Gehan-Breslow-Wilcoxon Test produced similar p-values for observed differences in survival.

6.3.4 SC-IN vaccination with 4-1BBL leads to improved survival compared with subcutaneous vaccination

We next examined the potential of 4-1BBL-NP LV to generate lung-based NP-specific T cell responses using subcutaneous priming and intranasal recall (SC-IN) as described in previous chapters. The resultant T cell response in BAL and PBMC separated from exsanguinated lung lysate was compared to that generated by Null-NP SC-IN vaccination and to dual subcutaneous vaccination with either 4-1BBL-NP or Null-NP. Mice were also challenged with lethal A/PR/8/34 as previously described.

Although there was a clear trend towards greater NP-pentamer positive CD8+ T cell responses in the lung lysate with SC-IN vaccination, these showed a much higher degree of variability in the SC-IN groups compared with SCx2 groups and consequently observed increase did not reach significance. In the BAL, however, SC-IN vaccination with either 4-1BBL-NP or Null-NP was significantly superior at inducing NP-specific CD8+ T cells whereas SCx2 vaccination induced negligible numbers of CD8+ T in this compartment.

Mice vaccinated SC-IN showed greater survival and less weight loss than was observed with subcutaneous vaccination. However, numbers of NP-specific CD8+ T cells isolated with a single 2mL BAL were not significantly different between 4-1BBL-NP and Null-NP SC-IN groups and there was no difference in survival or weight loss between these groups.

vFLIP-NP SC-IN was not included in parallel therefore absolute quantitative comparisons of NP-pentamer positive CD8+ T cells may not be valid. However, comparison of proportions of total lung CD8+ T cells that are NP-pentamer positive indicate that 4-1BBL-NP SC-IN generates a significantly lower proportion of NP-specific T cells in the lung than vFLIP-NP SC-IN (34.15% vs. 5.51%, p=0.0036).

It is unclear why the survival advantage of 4-1BBL NP SC vs. Null-NP SC is not observed with SC-IN vaccination. It may be the case that the marginally superior functional CD8+ and CD4+ systemic T cell responses after subcutaneous vaccination (but equivalent quantitative NP-specific CD8+ T cell response) seen with 4-1BBL NP over Null-NP are

relevant only in the context of post-challenge recruitment and expansion of T cells to the lung and airway, which may be accelerated by superior CD4+ T cell help and CD8+ memory cells with greater cytotoxic and proliferative potential. In the context of populations of lung-and airway- based CD8+ T cells present prior to challenge, however, this may be of less relevance. We could detect no difference in terms of functional surrogates (GzmB or Ki67) for cytotoxic or proliferative potential in lung-based T cell populations. As discussed in the previous chapter, the recall of T cells to the airway with vectors expressing antigen alone seems sufficient to substantially enhance GzmB production and Ki67 in these populations (4.3.5, page 158), and this increase may well overwhelm the more marginal differences seen between 4-1BBL-NP and Null-NP splenic CD8+ T cell populations seen in splenic memory populations.

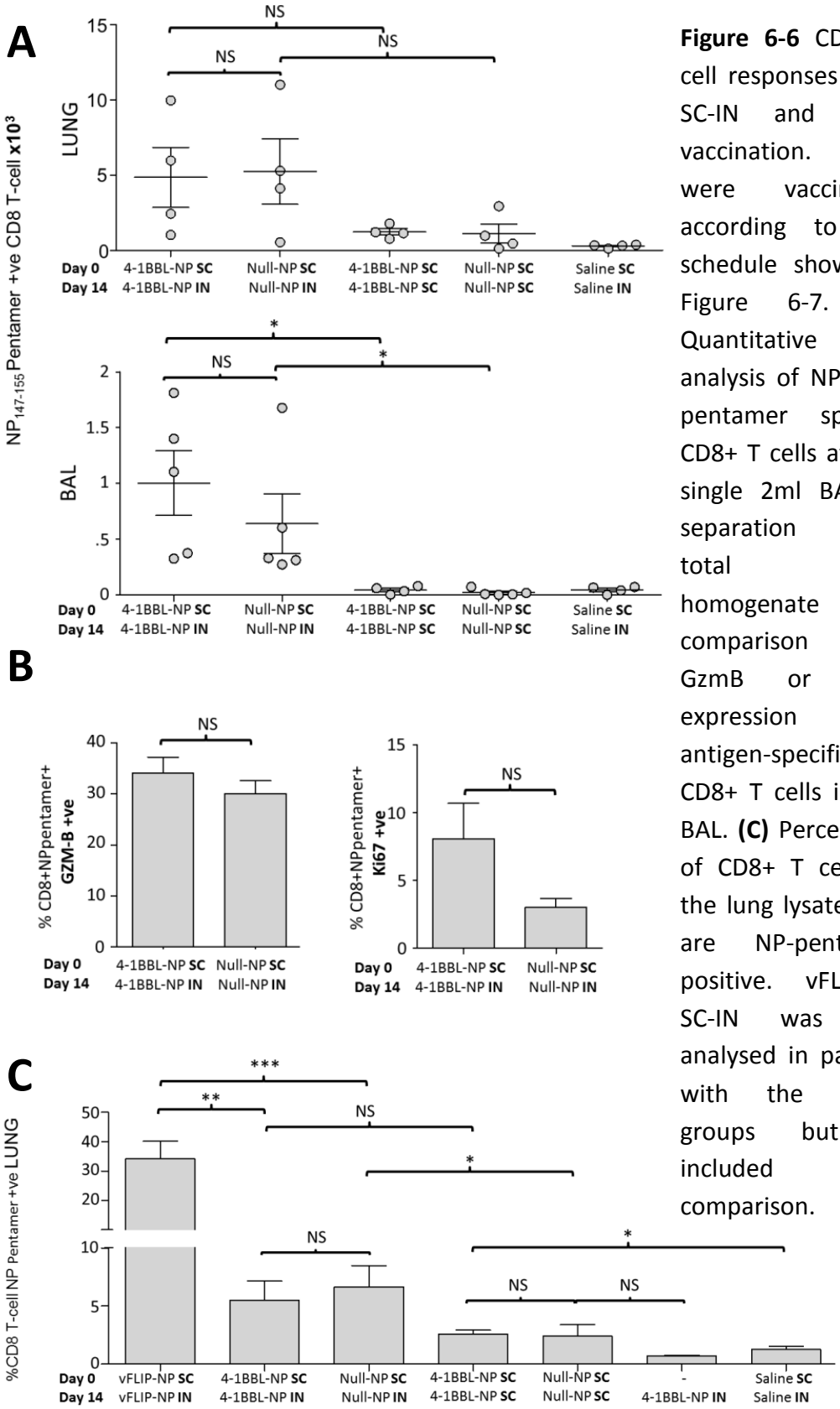


Figure 6-6 CD8+ T cell responses after SC-IN and SCx2 vaccination. Mice were vaccinated according to the schedule shown in Figure 6-7. **(A)** Quantitative analysis of $NP_{147-155}$ pentamer specific CD8+ T cells after a single 2ml BAL or separation from total lung homogenate **(B)** comparison of GzmB or Ki67 expression in antigen-specific CD8+ T cells in the BAL. **(C)** Percentage of CD8+ T cells in the lung lysate that are NP-pentamer positive. vFLIP-NP SC-IN was not analysed in parallel with the other groups but is included for comparison.

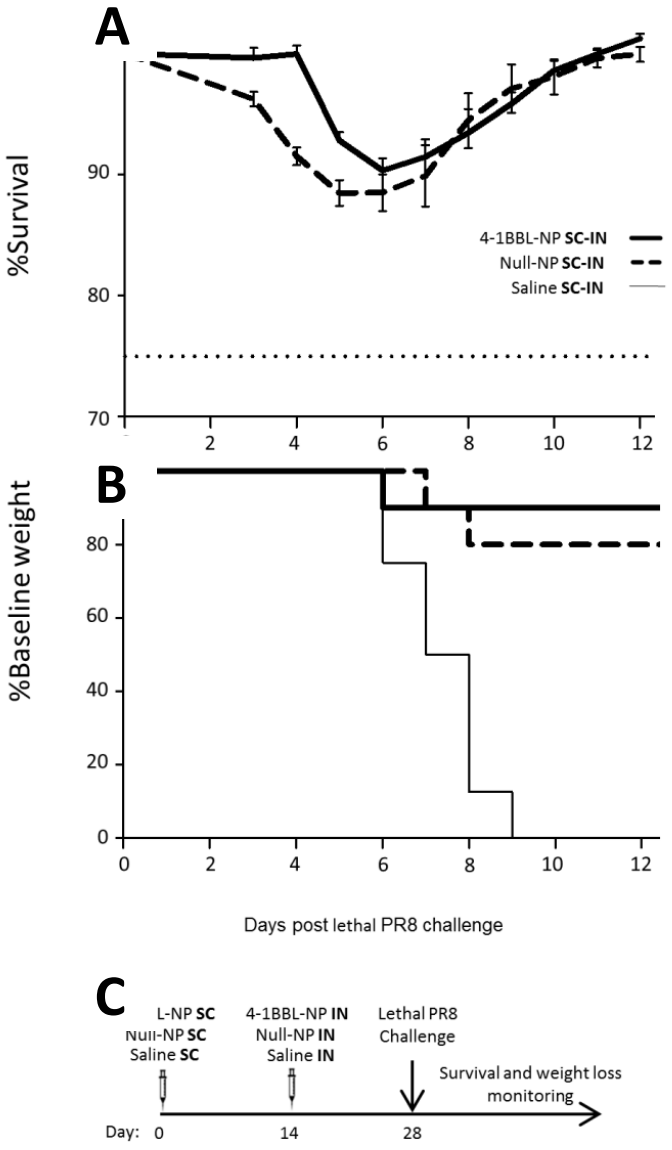


Figure 6-7 Weight loss **(A)** and survival **(B)** after lethal PR8 challenge of SC-IN vaccinated mice according to the vaccination schedule shown in **(C)**. No significant differences in survival or weight loss in survivors were observed.

6.3.5 4-1BBL has limited effects on T cell chemoattractant secretion by AM

We postulated that 4-1BBL-NP may generate inferior T cell recall responses in the lung compared with vFLIP-NP because transduction with 4-1BBL does not activate AM or induce chemokine secretion. We therefore examined the chemokine profile of BAL after intranasal administration of 4-1BBL-GFP as described previously. In addition, adherent cells from BAL were cultured for 4 days and chemokine levels measured in the supernatant thereafter.

Analysis of CD11c+F4/80+ AM in the BAL after administration of 200ng RT of 4-1BBL-GFP revealed equivalent rates of transduction (80%) as seen with vFLIP-GFP or Null-GFP and high specificity of transduction of this group (>99%). Correspondingly, transduced adherent cells that were cultured were exclusively of the AM phenotype.

Analysis of both BAL and cultured cell supernatants revealed relatively low levels of T cell chemokine production following intranasal 4-1BBL-GFP administration compared with vFLIP-GFP (Figure 6-8). IP-10, MCP-1 and MCP-3 were all found in the BAL at levels significantly higher than control or Null-GFP recipients. However, these were not detected in the supernatants of cultured adherent cells suggesting they do not originate from AM.

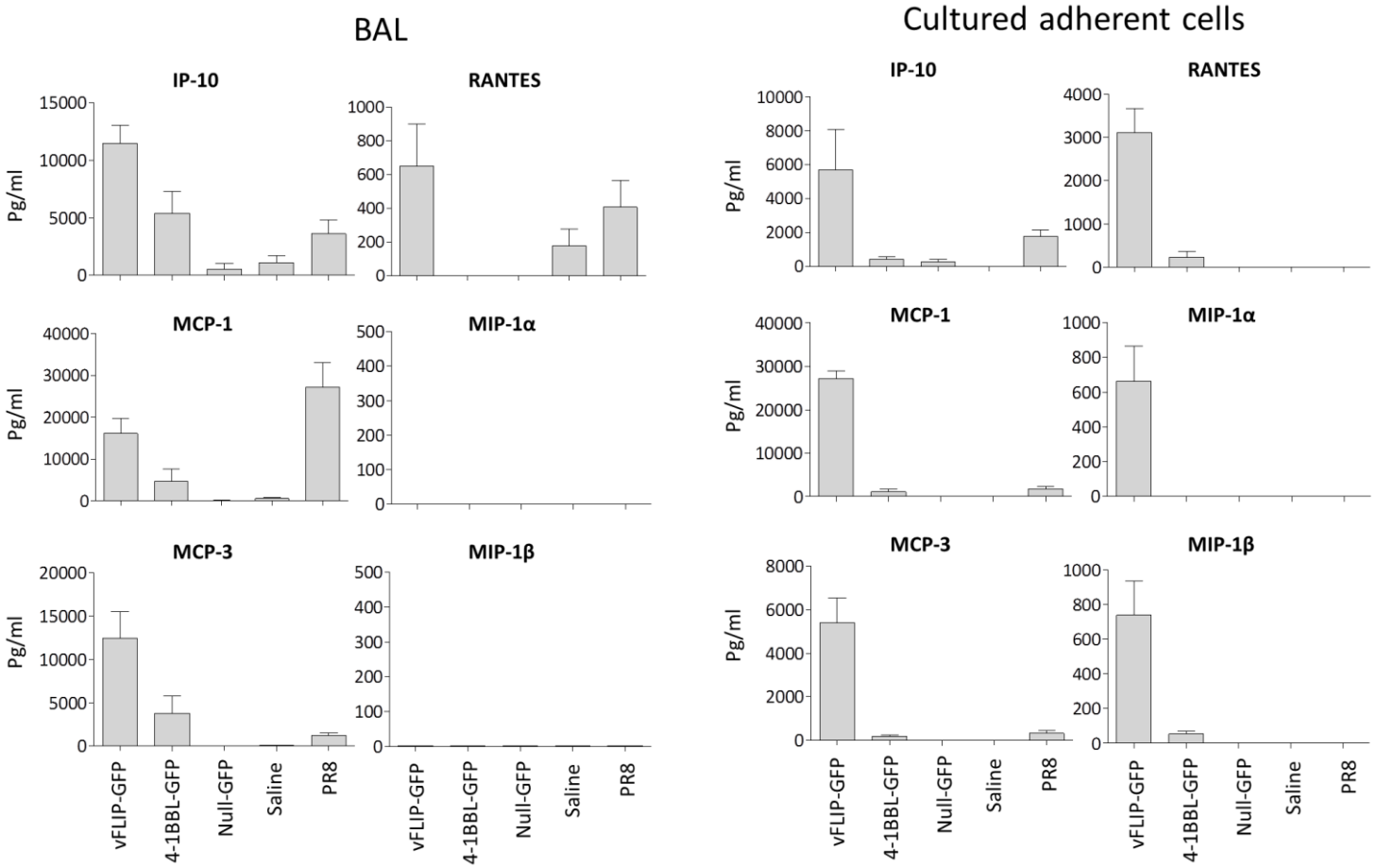


Figure 6-8 Analysis of chemokine levels in BAL and cultured supernatants (2 mL) of adherent cells from BAL following the same method as Figure 4-5 page 156. Cultured cells were phenotyped before and after culture by FACS, showing >99% of transduced cells were AM (F4/80+ CD11c+).

6.3.6 4-1BBL activates mouse bone marrow-derived DC, but the greatest activation is observed in untransduced bystanders

We postulated that overexpression of 4-1BBL may activate T cells indirectly by activating DC thereby increasing other DC co-stimulatory signals. DC activation has been shown to occur by both 4-1BBL reverse signalling and by stimulation through 4-1BB expressed on DC using monoclonal antibody or multimerised soluble 4-1BBL.

Using the same assay described previously for DC activation with vFLIP-GFP, we analysed expression of 4 activation markers (CD40, CD80, CD86 and ICAM-1) on DC following *in vitro* transduction with 4-1BBL-GFP.

This resulted in potent DC-activation, often surpassing that observed with LPS. However, activation markers were significantly more up-regulated on GFP- (untransduced) than GFP+ DC (Figure 6-9) within the target population in the same well. By contrast, vFLIP-GFP transduction resulted in minimal or no significant up-regulation of activation markers in untransduced cells and significant activation was only seen in the transduced population.

A number of mechanisms were hypothesised to explain the transactivation of untransduced DC:

1. 4-1BBL induced activation may occur through forward signalling via 4-1BB and 4-1BBL over expression down-regulates 4-1BB on the same cell.

2. 4-1BBL matures DC by reverse signalling at low levels of expression but that at high levels of transduction this signal becomes inhibitory.

3. 4-1BBL reverse signalling induces a cytokine that induces activation of bystander DC, but simultaneously down-regulates responsiveness to this signal in producer cells.

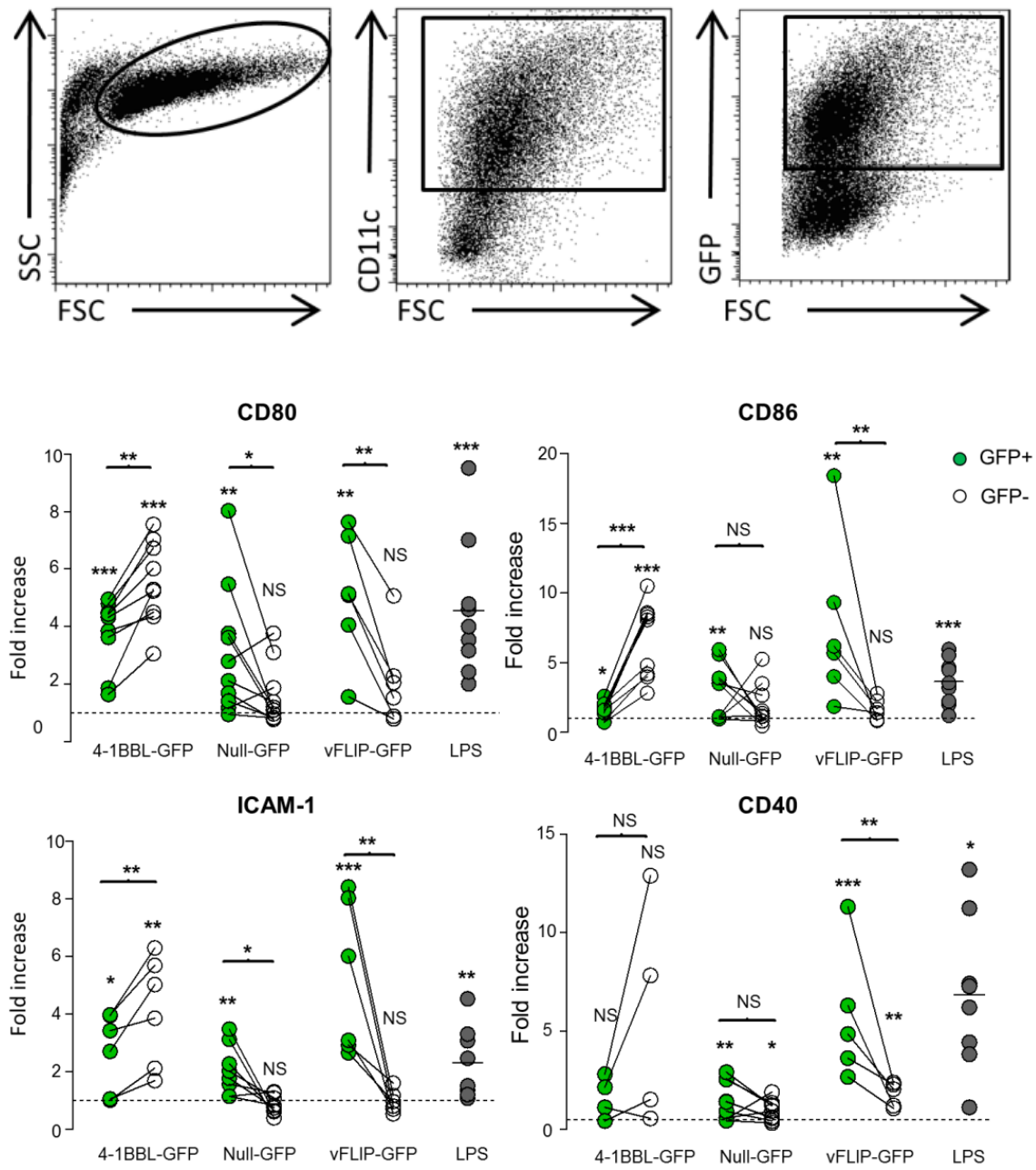


Figure 6-9 Analysis of four markers of DC activation at day 4 after transduction with 4-1BBL-GFP, Null-GFP or vFLIP. Expression of each marker is expressed as a factor of increase relative to untransduced DC (with the dotted line at 1 indicating no increase). Analyses of paired transduced (GFP+) cells and untransduced (GFP-) cells from the same well are joined by a connecting line. Each circle represents a separate experiment. T-tests comparing log(fold increase) were used to determine the p-value of observed differences between paired groups (significance level is shown over brackets) or whether the mean observed increase in expression is significantly greater than 1 (no increase) indicated by * over each population group. The gating strategy is shown above. NS= not significant.

6.3.7 4-1BBL DC transactivation is independent of reverse signalling, requires cell-cell contact and is abrogated by anti-4-1BBL antibody

To investigate the role of potential reverse signalling in DC maturation, we created a truncated mutant lacking the cytoplasmic N-terminal domain which includes two putative casein kinase I signalling regions. This mutant was expressed on the cell surface to an equivalent degree as wild-type (Figure 6-10). 3 independent repeats of the DC activation assay revealed stronger up-regulation of activation markers in the untransduced population as previously observed.

However, comparison between DC transduced with 4-1BBL WT and truncated 4-1BBL (4-1BBLTc) revealed the increase in activation markers was significantly higher in the WT group (Figure 6-11). This suggests that reverse signalling may play some role in activation of the transduced population. No significant differences were observed in DC activation in the untransduced populations in these groups.

Addition of 4-1BBL-GFP transduced DC to the upper well of transwell plates did not increase the activation of untransduced DC in the lower well, suggesting cell-cell contact is necessary for transactivation of DC by 4-1BBL, rather than a cytokine mediated mechanism (Figure 6-12). Furthermore, addition of anti-4-1BBL blocking antibody (clone TKS-1) consistently abrogated activation of the untransduced population in these experiments (Figure 6-11), regardless of whether 4-1BBLTc-GFP or 4-1BBL-GFP was used.

Taken together, these data strongly suggest that the DC activation observed in a total population of DC after addition of 4-1BBL-GFP occurs to a small degree by reverse signalling through 4-1BBL in transduced cells (with a contribution from LV-induced TLR stimulation as previously discussed), but to a much greater degree by forward signalling to untransduced bystander DC. This presumably occurs through 4-1BB receptor expression on mouse DC. Given that 4-1BB expression has been reported to suppress 4-1BBL expression, we postulated that the reverse may also be true, such that over-expression of 4-1BBL may suppress 4-1BB expression on the same cell, thus rendering the transduced population less responsive to 4-1BBL expression on neighbouring cells.

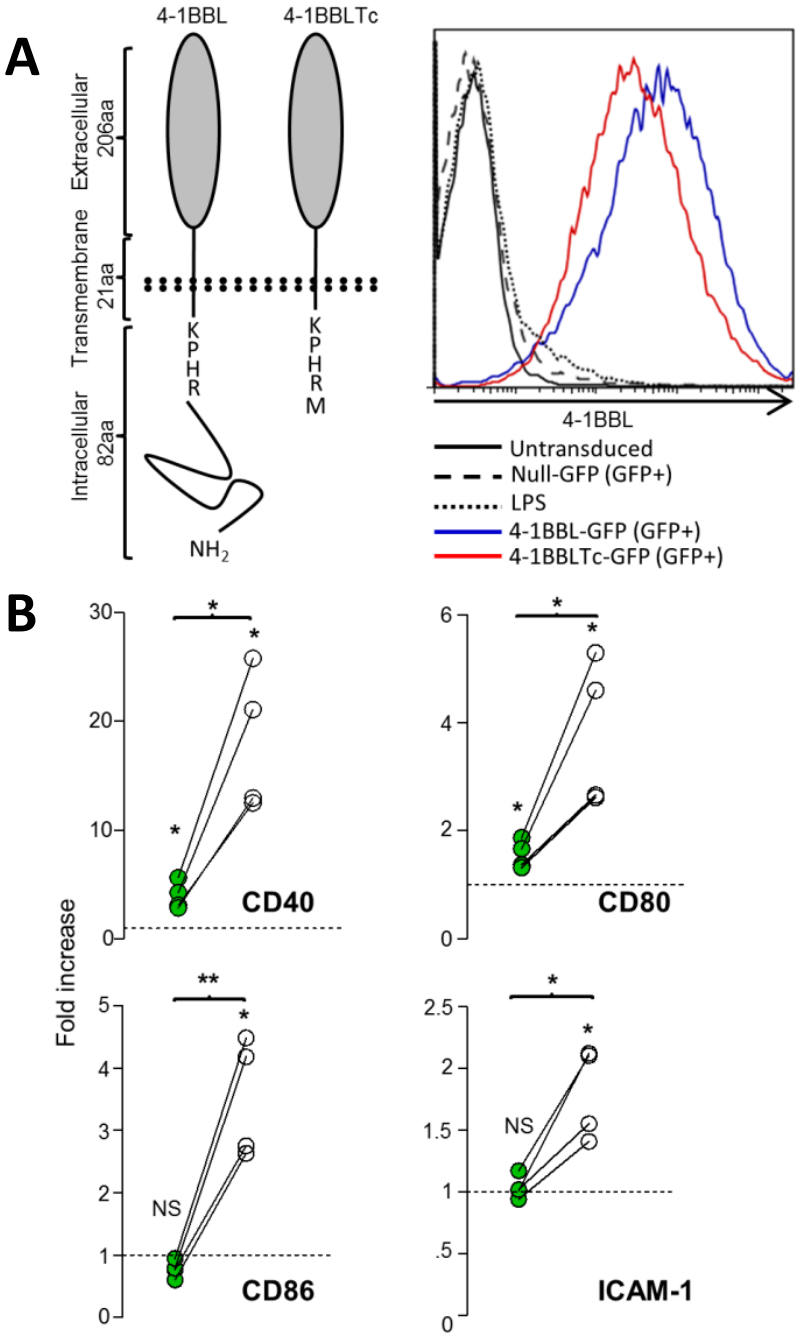


Figure 6-10 Truncation of 4-1BBL to remove the cytoplasmic N-Terminal domain (4-1BBLTc). This had no effect on expression level **(A)** or transactivation of untransduced DC in the target population **(B)**.

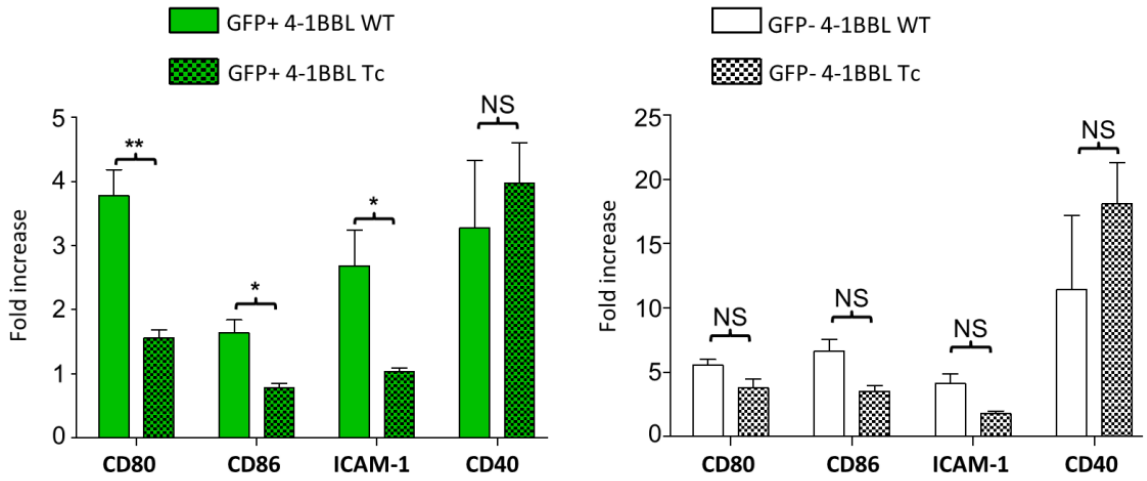


Figure 6-11 The degree of activation of transduced DC is significantly lower in DC transduced with 4-1BBLTc than wild-type. The degree of activation seen in the untransduced cells in target populations is not significantly affected by the N-terminal truncation.

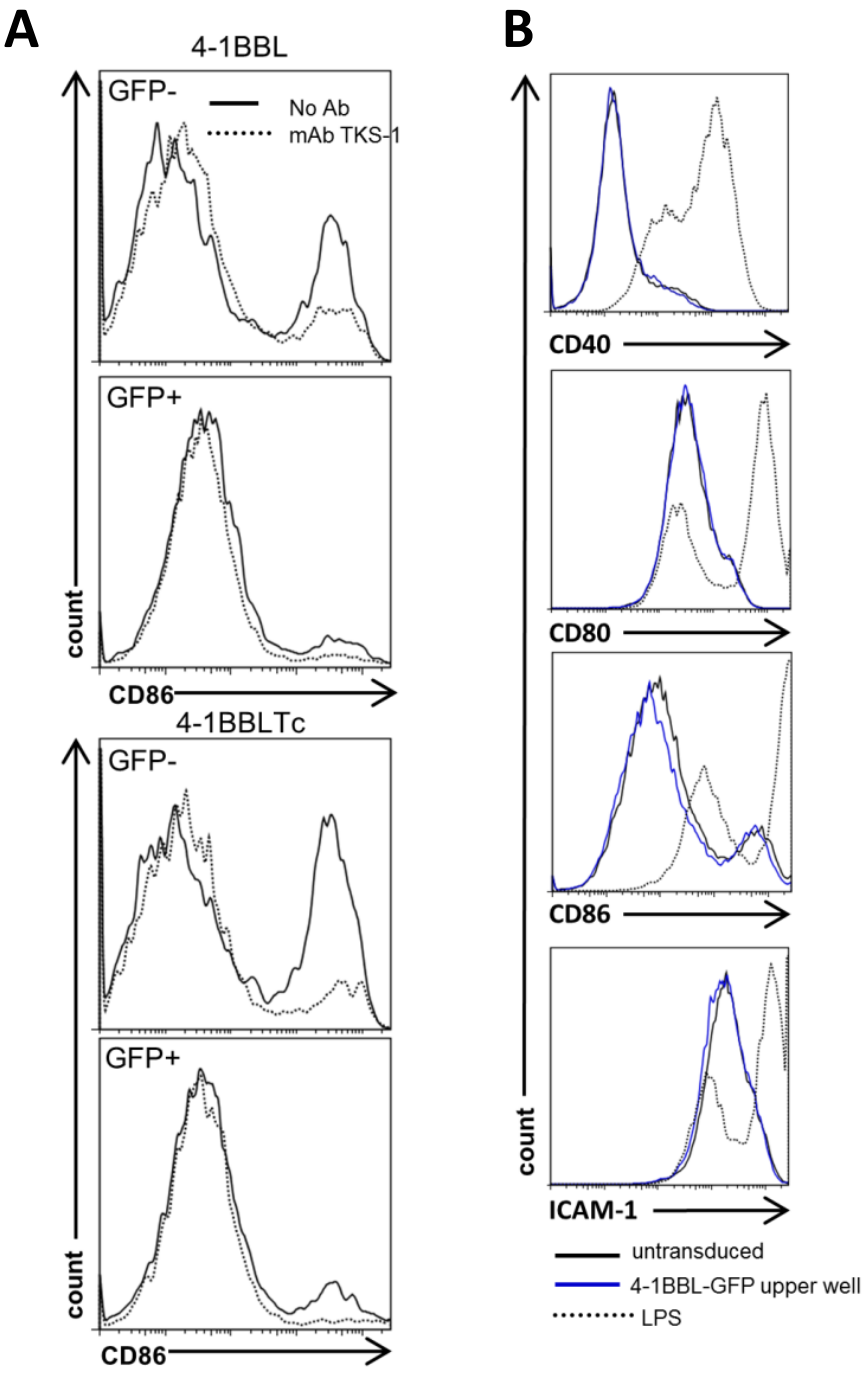


Figure 6-12(A) Transactivation of untransduced DC is abrogated in the presence of anti-4-1BBL blocking antibody clone TKS-1. This occurs in both 4-1BBL WT and Tc transduced populations. **(B)** In transwell experiments, addition of 4-1BBL transduced DC to an upper well separated from a lower well containing untransduced DC (by a 0.4um pore membrane) failed to induce their activation suggesting cell-cell contact is required for transactivation.

6.3.8 Membrane expression of 4-1BBL and 4-1BB are reciprocally controlled

4-1BB deficient mice express higher levels of 4-1BBL on the surface of LPS-activated DC, suggesting 4-1BB negatively modulates 4-1BBL expression. To examine the relationship between membrane expression of 4-1BBL and 4-1BB, bone-marrow derived murine DC were transduced with increasing quantities of 4-1BBL-GFP or 4-1BBLTc-GFP and extracellular and intracellular (after permeabilisation) of the ligand and receptor was analysed 2 days later.

Around 40% of untransduced mouse DC at day 6 post-maturation expressed 4-1BB. This 4-1BB+ve group were equally susceptible to transduction as 4-1BB-ve (Figure 6-13A) as evidenced by an equivalent proportion of GFP positive DC after addition of Null-GFP. However, following transduction with 4-1BB-GFP, 4-1BBL expression appeared to be restricted to the 4-1BB- population (Figure 6-13B – 1 ng RT/200000 DC) and was not expressed in 4-1BB+ transduced cells. Furthermore, as the amount of 4-1BBL-GFP expression increased with addition of higher concentrations of 4-1BBL-GFP, there was a steep decline in the proportion of DC expressing 4-1BB, eventually becoming undetectable on all cells at levels of maximal 4-1BBL-GFP transduction.

On intracellular staining however, with increasing 4-1BBL-GFP transduction, 4-1BB detection rose initially before a slight overall decline. Internalisation of 4-1BB is not an epiphenomenon of LV transduction itself, since increasing quantities of Null-GFP transduction have no effect upon 4-1BB membrane expression (Figure 6-14).

These data suggest restriction of 4-1BBL expression by surface 4-1BB at low MOIs, but if 4-1BBL expression is increased it can overwhelm this inhibition and instead down-modulate surface 4-1BB expression. Given that 4-1BB remains detectable intracellularly, it is feasible that down-regulation at the surface is achieved reciprocally by internal sequestration of 4-1BB by direct interaction with 4-1BBL.

A further possibility is that 4-1BB undergoes ligand-mediated down-regulation after contact with 4-1BBL expressed on other cells. However, the presence of a high concentration of anti-4-1BBL TKS 1 (sufficient to block staining with fluorochrome-conjugated antibody) had no effect on 4-1BB down-regulation as 4-1BBL transduction was increased (Figure 6-15A). Similarly, increasing concentrations of anti-4-1BB

stimulatory antibody (clone LOB-1) failed to induce down-regulation of 4-1BB (Figure 6-15B).

4-1BB down-regulation was also observed with transduction with 4-1BBLTc (Figure 6-13), suggesting that any interaction between 4-1BBL and 4-1BB leading to intracellular retention occurs between the extracellular or transmembrane domains of ligand and receptor.

This down-regulation of 4-1BB with increasing 4-1BBL expression would explain the observed differences in activation between transduced and untransduced DC seen in the same well after addition of 4-1BBL-GFP, since transduction with 4-1BBL-GFP would down-regulate 4-1BB on the same cell and inhibit responsiveness to 4-1BBL expressed on neighbouring cells. DC which have low levels of transduction or are untransduced would retain 4-1BB expression and undergo activation by neighbouring 4-1BBL transduced DC.

The simplest model to explain this phenomenon would be an interaction between 4-1BB and 4-1BBL intracellularly that prevents trafficking of both ligand and receptor to the membrane. A logical assumption would be that this occurs in the same way as 4-1BBL -4-1BB interaction at an intercellular synapse by canonical ligand-receptor binding at their extracellular domains, perhaps between ER-bound vesicles expressing both ligand and receptor. This “internal sequestration” mechanism would prevent both ligand and receptor from being expressed on the surface of the same cell, which would otherwise risk loops of sustained activation in the presence of similar DC. However, given that we could not detect 4-1BBL expression on untransduced DC and only very minor up-regulation with LPS stimulation on the 4-1BB negative population (data not shown) it seems likely that a scenario in which 4-1BBL is overexpressed to the degree that 4-1BB is suppressed is not physiologically relevant, and this mechanism probably serves to limit ligand expression on receptor positive DC only.

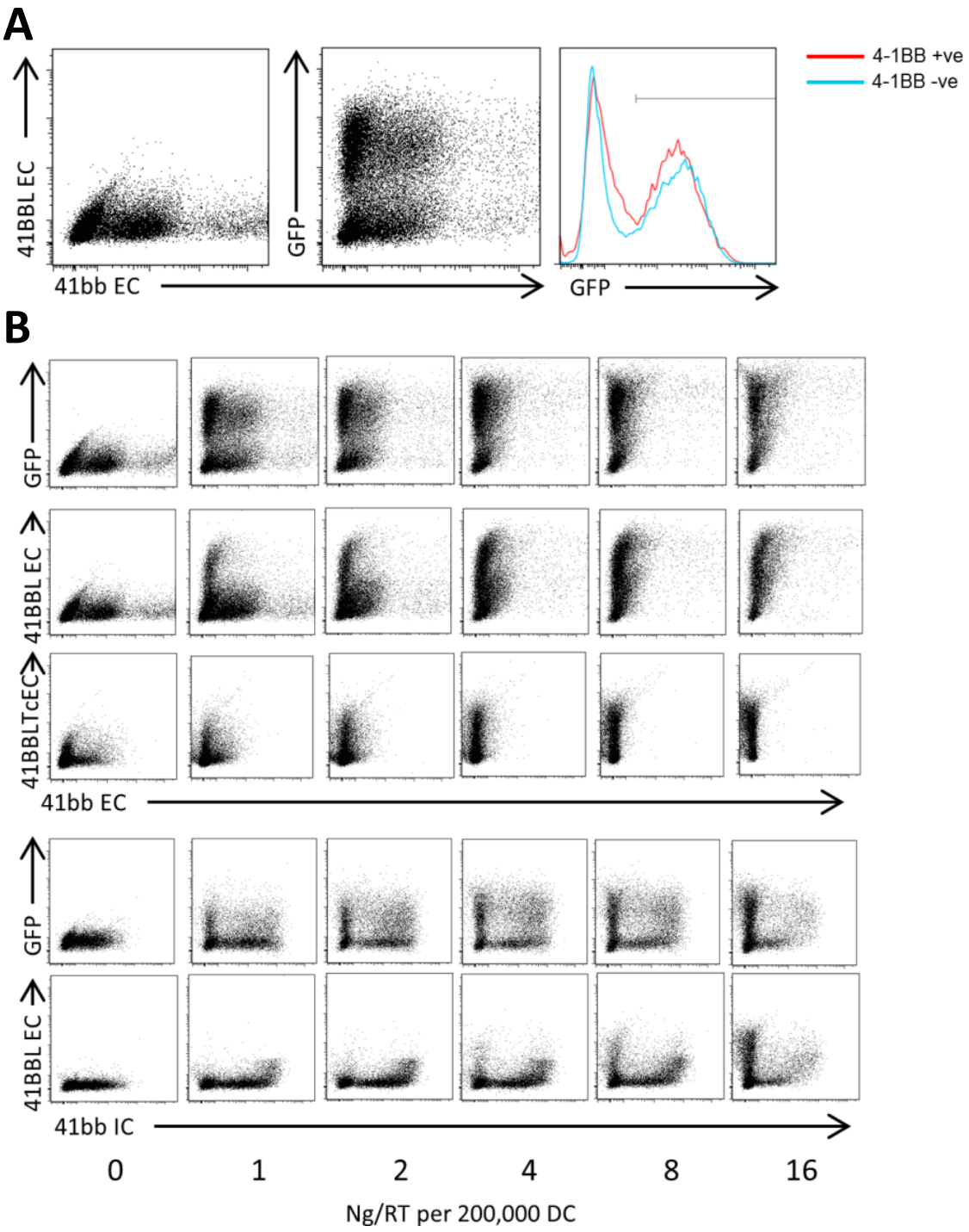


Figure 6-13 Reciprocal limitation of expression of 4-1BBL and 4-1BB on bone-marrow derived mouse DC. **(A)** DC express 4-1BB but not 4-1BBL. Both 4-1BB+ and 4-1BB- are transduced by LV with equal efficacy (histogram). **(B)** 4-1BBL expression is suppressed in 4-1BB+ cells but as MOI is increased 4-1BB expression is supressed by increasing 4-1BBL expression. This also occurs with increasing expression of 4-1BBLTc. Intracellular 4-1BB remains detectable suggesting suppression of extracellular expression only.

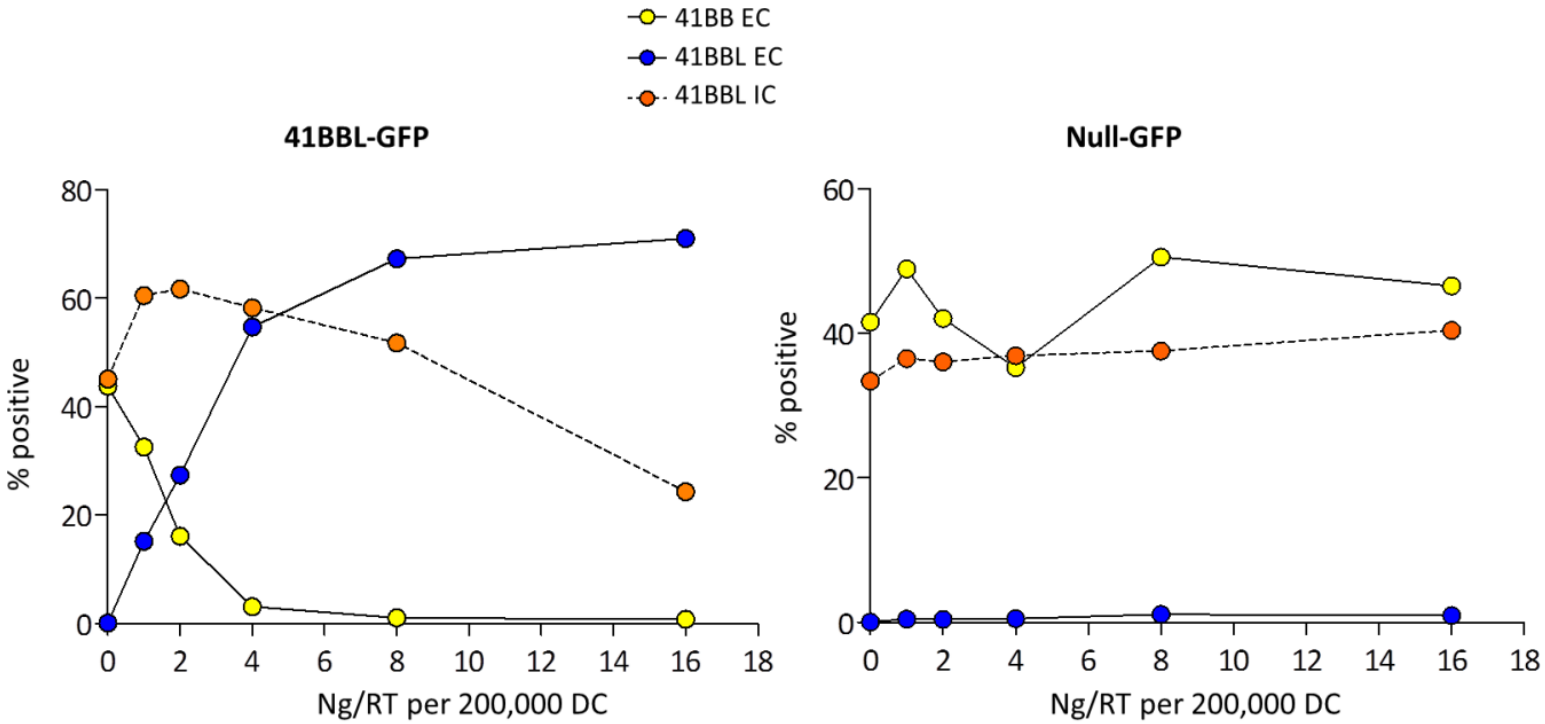


Figure 6-14 Interrelationship of extracellular and intracellular expression of 4-1BBL and 4-1BB derived from FACS plots in Figure 6-13. For comparison, data is shown with increasing transduction of DC with Null-GFP, which does not alter 4-1BB or 4-1BBL expression.

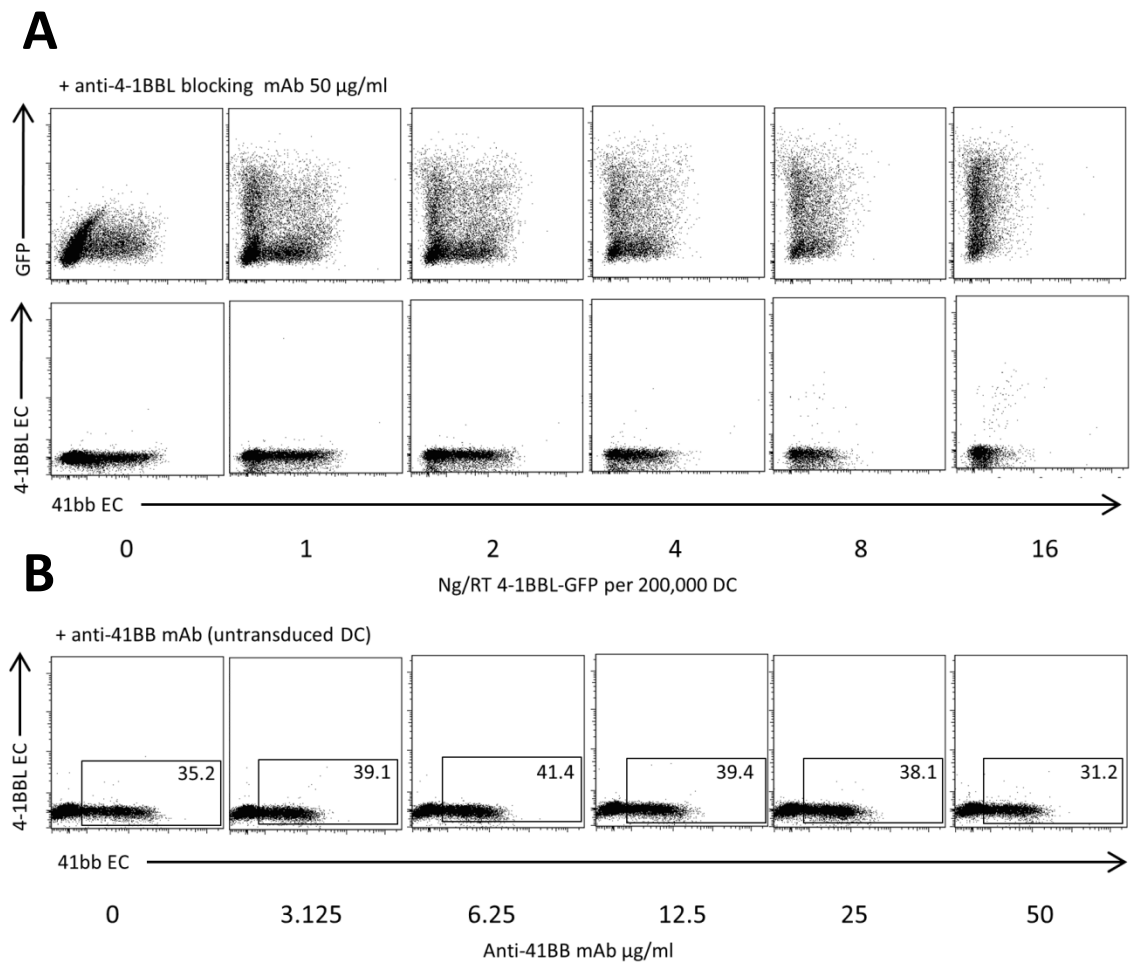


Figure 6-15 Blocking 4-1BBL-4-1BB interaction has no effect on reciprocal down-regulation **(A).** Stimulation/crosslinking of DC with anti 4-1BB mAb does not induce 4-1BB down-regulation **(B).**

6.3.9 4-1BBL transactivation of DC occurs *in vivo* in BALB/c and Bl/6 mice

To test whether DC transactivation occurs *in vivo*, we injected groups of 4 mice at separate sites with different vectors in the flank and hindquarter and then collected draining inguinal lymph nodes (Figure 6-16). The original intention was to inject at site A with a vector expressing Thy1.1 only, and then at site B with either 4-1BBL-GFP or Null-GFP. Thy1.1-expressing DC in the lymph node would then be analysed for activation markers to determine whether 4-1BBL-GFP *in trans* may enhance their activation. However, Thy1.1 +ve DC were detectable in small amounts (<100) in draining LN at day 4 which made meaningful analysis difficult. We therefore examined total GFP- DC in draining lymph node at day 4. This revealed both greater numbers of CD11c+ MHCII+ GFP- cells in the draining lymph node of mice receiving 4-1BBL *in trans* vs Null-GFP *in trans* and higher numbers of DC expressing CD80, CD86 or both. 4-1BBL thus appears to both recruit and activate DC in draining lymph nodes. To further characterise the degree of up-regulation of CD80 and CD86, BL/6 mice were injected but lymph nodes pooled between groups of 4 mice and CD11c+ cells isolated by bead separation. Cells were then stained for CD11c, MHC II and CD80 or CD86.

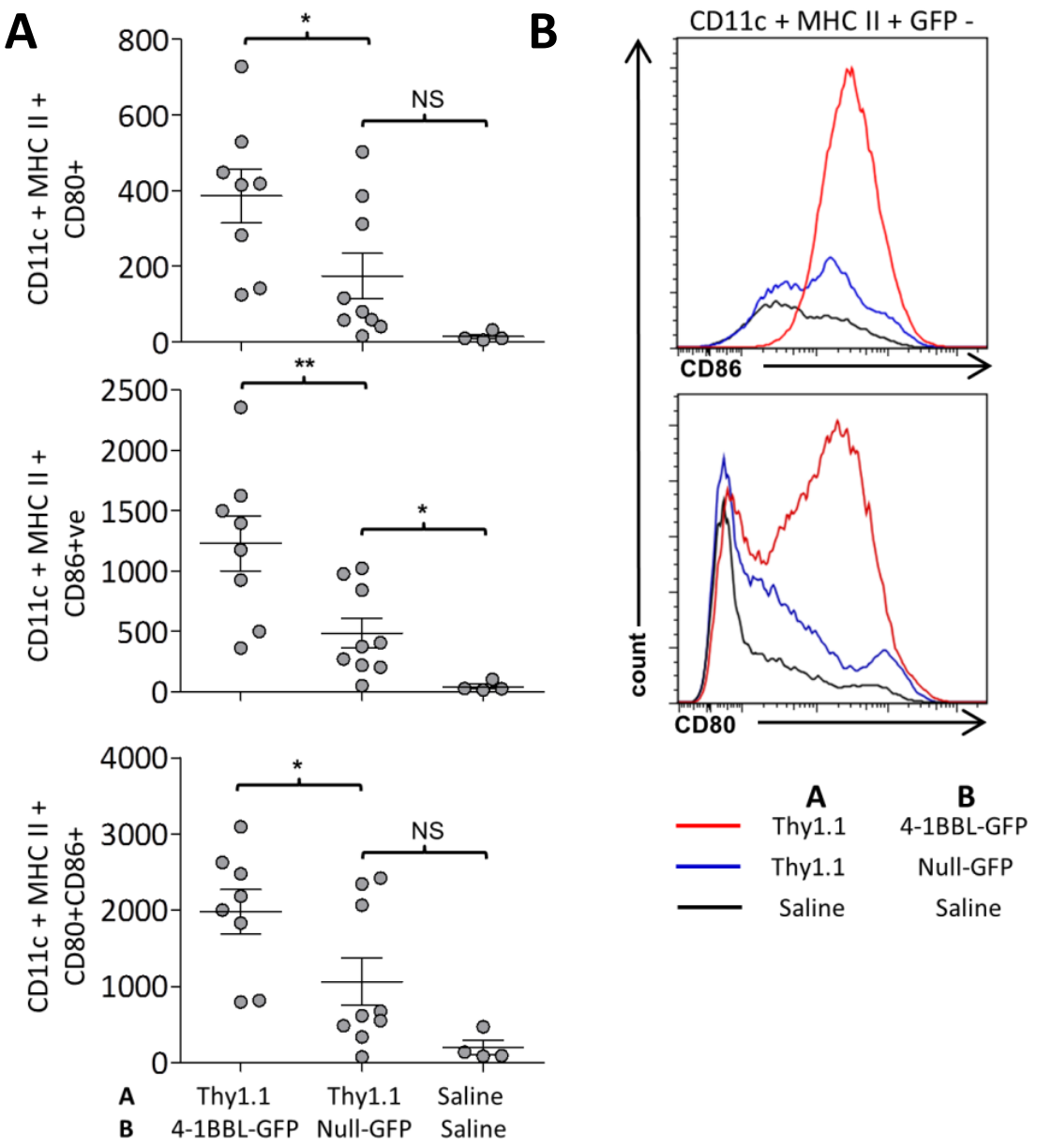
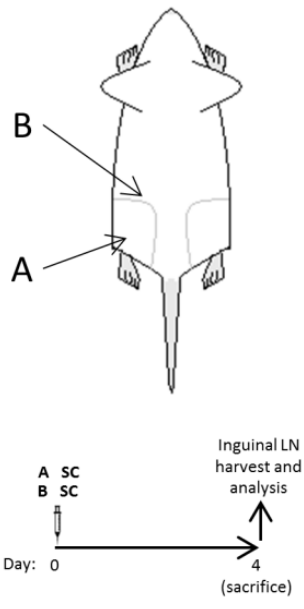


Figure 6-16. 4-1BBL LV induce transactivation of untransduced DC *in vivo*. **(A)** Mice were injected with a Thy1.1 expressing LV at site A and either 4-1BBL-GFP or Null-GFP at site B, both of which drain to the inguinal lymph node. On Day 4, Lymph node cells were harvested and stained for CD11c, MHC II, CD80 and CD86. **(B)** Analysis of degree of expression of CD80 and CD86 in untransduced cells from lymph nodes pooled from 4 mice and CD11c+ cells isolated by magnetic bead separation.



6.3.10 4-1BBL enhances antigen-specific CD8 T cell function when expressed *in trans*

Reciprocal control of 4-1BB by 4-1BBL raises a fundamental problem with the use of 4-1BBL as a vectored adjuvant co-encoded with antigen. We have shown here that potent transactivation of DC occurs with 4-1BBL overexpression and this may account for a significant proportion of the observed enhancement of T cell responses together with direct stimulation of 4-1BB on T cells. The use of 4-1BBL encoded *in cis* with antigen, however, means that transduced DC will themselves be insusceptible to 4-1BBL mediated transactivation due to 4-1BB down-regulation. This would potentially limit the activation status of transduced DC and thus co-stimulation provided to T cells. This implies that 4-1BBL may be a more effective adjuvant if expressed *in trans* with antigen.

To test this, groups of 4 mice were vaccinated subcutaneously on one side in two separate sites which drain to the inguinal lymph node. These combinations of vaccinations provided 4-1BBL *in cis* and *in trans* with antigen and are shown in Figure 6-17. Lymph nodes and spleens were harvested and T cell responses were then assessed 14 days later.

$NP_{147-155}$ pentamer responses in the spleen were equivalent between all 4 groups of vaccinated mice. However, mice receiving 4-1BBL-GFP *in trans* with Null-NP demonstrated higher GzmB expression in CD8+ T cells after overnight stimulation with peptide than was observed in mice receiving 4-1BBL-NP and Null-GFP in trans. In the lymph node, the mice receiving 4-1BBL-GFP *in trans* with Null-NP were the only group to demonstrate significantly greater GzmB expression upon re-stimulation compared with mice vaccinated with Null-NP and Null-GFP *in trans*.

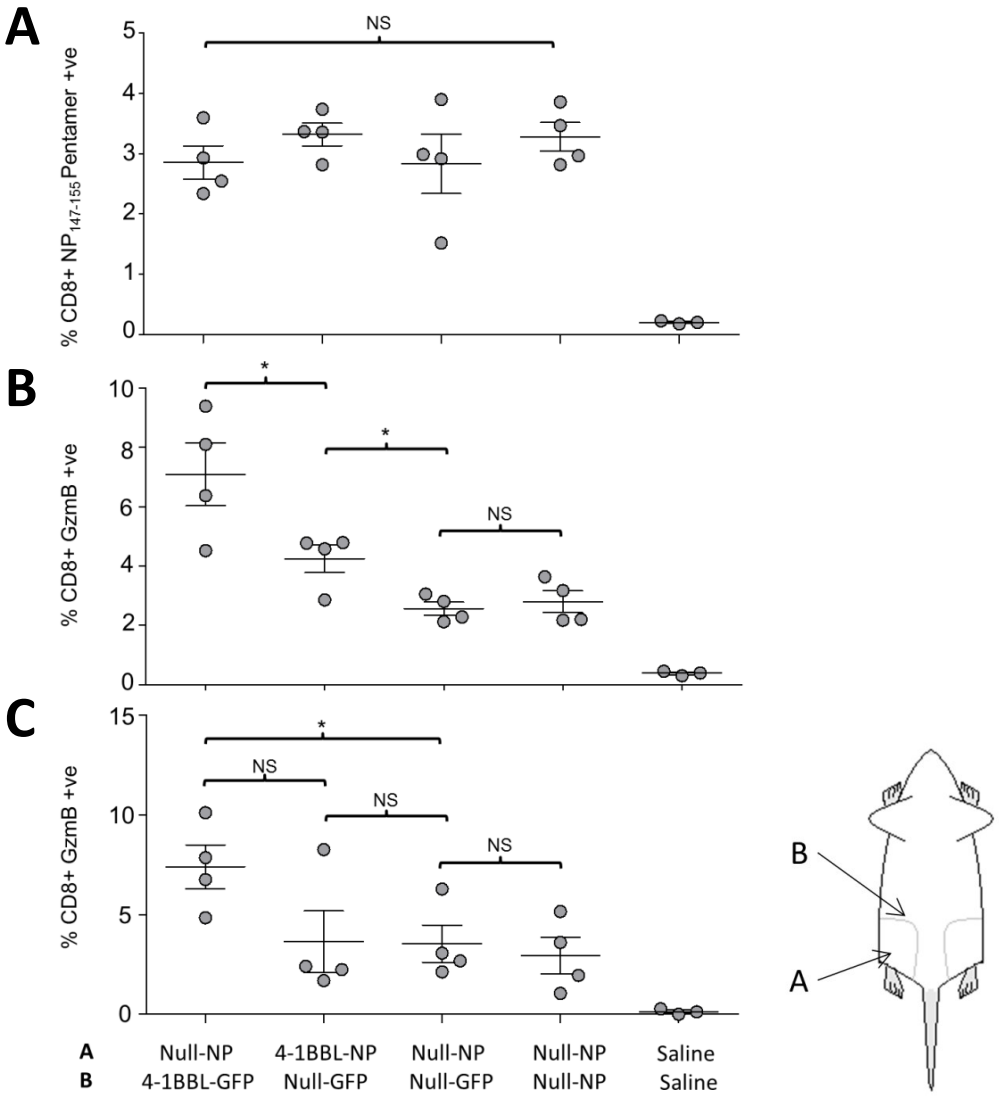


Figure 6-17 4-1BBL enhances the quality of the CD8+ T cell response when expressed *in trans* Mice were immunised at 2 sites in the flank with lentiviral vectors then analysed after 14 days. The percentage of $NP_{147-155}$ pentamer+ CD8+ T cells (of total CD3+CD8+) in spleen **(A)** GzmB+ CD8+ T cells in spleen **(B)** and granzyme B+ CD8+ cells in the draining lymph nodes **(C)** are shown.

6.4 Summary

LV co-expressing 4-1BBL and NP induced superior functional CD8+ and CD4+ T cell responses against NP following subcutaneous vaccination compared with LV expressing NP alone. We did not observe significant differences in numbers of $NP_{147-155}$ pentamer positive CD8+ T cells generated by 4-1BBL-NP compared with Null-NP vaccination, although it is possible that analysis at 14 days is too early to detect any prolongation of memory T cell survival conferred by 4-1BB stimulation. Vaccination with 4-1BBL-NP confers greater protection against lethal A/PR/8/34 challenge than vaccination with Null-NP, but 4-1BBL-NP and Null-NP induce equivalent lung- and airway-based T cell numbers and protection when given intranasally after subcutaneous priming. Unlike vFLIP, 4-1BBL does not induce significant chemokine secretion in AMs.

4-1BBL-GFP induces *in vitro* mouse DC maturation in transduced cells, but activation is greater in untransduced cells in the same target population. Transactivation of untransduced cells is blocked by anti-4-1BBL antibody and requires cell-cell contact and 4-1BBL also transactivates DC *in vivo.* 4-1BBLTc-GFP has an equivalent effect upon untransduced cells, but transduced DC are matured to a lesser degree than with 4-1BBLWt. Membrane expression of 4-1BBL and 4-1BB are reciprocally controlled in DC. Whilst LV encoded 4-1BBL is an effective adjuvant when expressed *in cis* or *trans* with antigen, expression *in trans* may result in functionally superior T cell responses.

6.5 Discussion

Despite the widespread experimental use of 4-1BB stimulation to enhance anti-tumour T cell responses, few studies have attempted to isolate the degree to which this occurs through 4-1BB expressed on T cells or DC. Zhu *et al* have addressed this by adoptively transferring CD8+ T cells from 4-1BB KO (Thy1.2+) mice into recipients and then stimulating with anti-4-1BB mAb *in vivo*. They found expansion of adoptively transferred WT CD8 + T cells but not 4-1BB deficient T cells. However, this experiment did not involve antigen, therefore the duration of any co-stimulation of the T cell response through 4-1BB activated DC would be abbreviated by the absence of concurrent antigen-presentation and MHC-TCR interaction. A further confounder is that 4-1BB deficient T cells do not behave normally (irrespective of 4-1BBL stimulation) compared to WT, undergoing hyperproliferation in the absence of TCR stimulation and reduced cytokine secretion, phenomena which are as yet unexplained.

A growing number of studies have observed high 4-1BB expression on maturing DC and DC activation by either anti-4-1BB mAb or transfected ligand[567,621,622]. Triggering 4-1BB signalling on DC results in enhance IL-6 and Il-12 secretion, up-regulation of CD80 and CD86 DC and enhanced allogeneic mixed lymphocyte reactions[623] and 4-1BB deficient DC exhibit poorer survival and are less able to sustain CD4+ T cell responses to pulsed antigen[624].

Here we have shown that 4-1BB mediated DC activation by 4-1BBL up-regulation on adjacent DC, and a lesser degree of activation by reverse signaling through the over-expressed ligand. *In vivo* subcutaneous injection of LV expressing 4-1BBL appears to recruit large numbers of untransduced, activated DC to the local lymph node. This non-specific transactivation of DC may have a number of physiological roles. The traditional paradigm of antigen presentation by dendritic cells involves uptake of antigen in the periphery and migration to local lymph nodes for presentation of processed antigen to naïve T cells. This is often known as the Langerhans cell paradigm, since skin-derived DC are the most extensively studied DC subset in the context migration and T cell priming. However, the identification of multiple subtypes of dendritic cell with distinct homing, life-cycle and antigen presenting capabilities has challenged this model[625–627].

Importantly, CD8+ lymphoid resident DC have been shown to be capable of cross-presenting antigen transferred from incoming migratory DC. Indeed, in some models of infection (such as dermal HSV-1 infection) Langerhans DC do not directly prime naïve T cells after migration to local lymph nodes, a function instead performed by CD8+ lymph node-resident DC (LN-DC) after antigen transfer.

The mechanism of antigen transfer between DC remains unknown. Furthermore, the means by which migratory DC, having been activated by inflammatory mediators at the site of infection, pass on this activation pattern to recipient LN-DC is also unknown. This will partly be achieved by the transfer of antigen and therefore stimulation of the same pattern recognition receptors to which the migratory DC was subjected, provided the antigen is sufficiently intact. However, LN-DC will not be exposed to antigen-independent activatory signals present only at the site of infection. A mechanism must therefore exist whereby migratory DC transduce these signals to antigen recipient DC in the lymph node. Some have proposed this occurs indirectly through activation of lymph node CD4+ T cells by migratory DC and subsequent licensing of LN-DC by T-helper cytokines such as IL-4[628].

Mouse splenic and bone-marrow derived DC constitutively express high levels of 4-1BB and are therefore susceptible to 4-1BBL induced activation. 4-1BBL is only expressed on DC following stimulation with, for example, anti-CD40 antibody or LPS[629]. We may therefore postulate a mechanism whereby LN-DC are directly transactivated by incoming, activated migratory DC. This interaction may also reinforce activation of the instigator by reverse signalling through 4-1BBL. However, 4-1BBL expression in 4-1BB^{HI} transactivated LN-DC would risk propagation of activation to DC bearing other unrelated antigens or self-antigens. We have demonstrated that even when over-expressed through strong viral promoters in transduced murine DC, 4-1BBL expression at the membrane is limited to the 4-1BB negative DC population. Likewise, 4-1BB expression can be overwhelmed by 4-1BBL transduction. This stringent reciprocal regulation of 4-1BB and 4-1BBL would mitigate the risk of 4-1BBL/4-1BB mediated propagation of PRR-independent activation through a DC population.

This may also explain our observed results that 4-1BBL is more effective at generating GzmB T cell responses against LV-encoded antigen *in trans* rather than *in cis,* which may be of importance in the context of using 4-1BBL as a vectored adjuvant.

4-1BBL NP SC-IN vaccination did not induce larger airway or lung-based NP-specific T cell populations than Null-NP SC-IN and this corresponded to poor induction of a T cell chemoattractant response by AM to 4-1BBL-GFP transduction. We found that AM, unlike mouse bone-marrow derived DC, did not express 4-1BB which may explain the absence of AM activation by this approach. Correspondingly, 4-1BBL-NP SC-IN vaccination showed no benefit over Null-NP SC-IN in terms of surrogates of T cell function or protection against challenge. However, we did not examine the longevity of the T cell response induced by 4-1BBL-NP versus Null-NP. Given that 4-1BB stimulation enhances survival of T cells, longer term analysis may reveal advantages of 4-1BBL-NP over vaccination with LV expressing antigen alone. This raises the interesting question of whether lung-resident T cell responses are more effectively maintained dynamically, by ensuring a prolonged T cell chemo attractant signal and antigen expression, or statically by increasing resident T cell memory survival.

In summary, expression of 4-1BBL in LV enhances T cell responses and protection in an influenza model against co-encoded antigen, but a significant share of this activation may be attributable to transactivation of DC. Exploring the relative contribution of direct and indirect T cell stimulation via 4-1BB has previously been approached by use of 4-1BB and 4-1BBL knockout models which are confounded by abnormal T cell proliferation and function in the absence of 4-1BB stimulation. In future work we aim to address this issue by vaccination with LV co-encoding shRNA against 4-1BB together with antigen, co-injected with 4-1BBL LV *in trans* at a separate site draining to the same lymph node. Given that mouse T cells are resistant to LV transduction, any reduction to the adjuvantic effect of 4-1BBL given *in trans* could then be attributed to the shRNA mediated loss of 4-1BB expression in DC transduced with antigen and consequent diminished DC activation.

7 Conclusions, limitations and future directions

This study set out to test the efficacy of lentiviral vectors as vaccines for the generation of T cell responses against acute viral infection. Influenza was chosen as a disease model not only because it provides a robust challenge for this potential vaccine modality but also because, as the recent SOH1N1 pandemic revealed, it is a highly relevant example of a fast-mutating, multi-subtype infection for which there is a pressing need for a universal vaccine.

We evaluated the adjuvantic potential of the NFκB-activator, vFLIP, and stimulation of the 4-1BBL:4-1BB signalling axis. Both approaches yielded unexpected mechanisms by which these molecules may enhance T cell responses to LV vaccination. For example, vFLIP-mediated NFκB stimulation of AM failed to prime T cell responses against co-transduced antigen, but generated a potent chemokine response that enhanced recall of pre-existing NP-specific T cells to the lung, even when originating from small numbers of adoptively transferred AM. 4-1BBL expression strongly transactivated DC through 4-1BB, an important indirect pathway contributing to T cell activation.

These findings have underscored the utility of two attributes of LV: the ability to transduce non-dividing cells with high efficiency which permits manipulation of a range of immune cells accessed by different routes, and their intrinsic low immunogenicity, which allows discernment of the effects of co-encoded transgenes. These attributes underlie the significant potential of LV both as clinical vaccines and also as immunological tools for unravelling molecular interactions between immune cells.

7.1 LV vaccines as immunological tools

LV are currently the most popular modality for experimental gene transfer or knockdown (typically encoding short-hairpin RNA, shRNA) of targets for both *in vitro* and *in vivo* use. Substantial investment by the life sciences industry mean mouse and human cDNA expression libraries are available in lentiviral backbones and LV

expressing multiple candidate shRNAs against a knock-down target of choice can be purchased “off-the-shelf”. As discussed previously, LV can then be pseudotyped in a variety of ways to target particular cell types. In this study we have examined the relatively simple approach of over-expression of chosen immunogenic adjuvants using an LV with a broad tropism, but have not explored the potential of knocking-down gene expression, or targeting particular cells of the immune system. There are a number of limitations and questions raised in the work presented here which may be readily addressed with experiments that exploit the full functional range of LV.

Is transduction of alveolar epithelial cells with vFLIP-NP sufficient to induce T cell recall to the lung?

We have shown that LV-transduced AM are sufficient for T cell recall to the lung, even in small quantities, but we have not conclusively demonstrated that they are necessary. This is not trivial, since a central hypothesis arising from this work is that chemokine production by AM is pivotal to the potency of LV as mucosal T cell vaccines. To confirm this would requires exclusion of transduced epithelial cells as a major source of T cell chemoattractant after intranasal vFLIP-NP. We have developed a trivalent LV that expresses two transgenes under the control of one promoter (SFFV), separated by an internal ribosome entry site (IRES) and another under a separate promoter. An LV with the DTR receptor driven by an F4/80 promoter and vFLIP/antigen under the SFFV IRES cassette could be used to selectively kill transduced, F4/80 positive cells with intranasal diphtheria toxin, leaving transduced epithelial cells intact. This avoids the confounding, pro-inflammatory consequence of AM depletion by clodronate or diphtheria toxin in the CD11C-DTR mouse model.

Is antigen or T cell chemoattractant (or both) required to maintain lung based T cell memory?

A central question in immunology is whether persistent antigen presentation is required to maintaining tissue-resident T cell populations in the longer term. A trivalent LV expressing the DTR receptor could also be used to “switch off” antigen

(and/or expression of a chosen chemokine) at a chosen point after intranasal boosting by administration of intranasal and/or intravenous diphtheria toxin.

What are the functional and quantitative requirements for T cell mediated protection against influenza viral challenge?

Despite the widespread reliance upon splenocyte IFNγ ELispot, pentamer/tetramer quantification and splenocyte cytotoxicity assays in the literature as surrogates of T cell vaccine efficacy, the relative importance of quantitative and qualitative T cell responses in protection against influenza remains unclear. It is noteworthy that neither vFLIP nor 4-1BBL co-expression with antigen significantly increased the magnitude of NP-specific CD8+ T cell responses to SC vaccination compared with LV expressing NP alone. Functional markers (such as IFNγ secretion upon re-stimulation and GzmB expression) and survival after lethal challenge were significantly improved by these adjuvants, but the greatest enhancement in the degree of protection was observed by the SC-IN prime-boost strategy. Even using LV expressing NP alone, SC-IN vaccination conferred substantially greater survival and less weight loss than SC vaccination with vFLIP-NP or 4-1BBL-NP. This would suggest that the spatiotemporal numbers of T cells generated by vaccination are far more important than functionality. However, SC-IN Null-NP vaccination, even in the absence of vFLIP, generated GzmB-rich T cell populations not found in the spleen after SC vaccination. This indicates functional modification of memory T cells upon recall to the lung. One shortcoming of this work is that is unclear to what degree the increased GzmB expression observed in airway and lung NP-specific T cells after vFLIP-NP SC-IN vaccination compared Null-NP SC-IN is due to functional programming at priming (by vFLIP-transduced DC) or functional enhancement at recall (by antigen or other signals from vFLIP-activated AM or epithelial cells). It is also unclear from these data whether it is the superior numbers of lung and airway NP-specific CD8+ T cells or superior GzmB content (or indeed both) that determine the superior protection conferred by vFLIP-NP SC-IN vaccination over Null-NP SC-IN. These questions could be readily addressed by cross-over combinations of vaccination with vFLIP-NP and Null-NP, SC and IN.

This is an important issue to resolve, since little is known about the quantitative and functional thresholds that must be met for an effective mucosal T cell vaccine against influenza and other respiratory pathogens. The recent finding of Lambe *et al*, that large numbers of IFNγ secreting lung-based CD8+ T cells could be generated by an NP+M1 Adenovector-MVA SC-IN vaccination (~30% of total CD8+) is of interest because this failed to confer significant protection against influenza challenge with A/PR/8/34[464]. This mirrors the relatively weak cytokine responses we observed in lung and airway T cells after SC-IN vaccination to peptide re-stimulation despite complete protection. It is tempting to assume cytotoxic function, indicated by high GzmB staining, is therefore the more important requirement for effective protection. However, a limitation of this work is that we have not definitively shown this with cytotoxicity assays. *In vitro* assays would not replicate the immunotolerant environment of the airway, and attempts to deliver target cells intranasally for an *in vivo* measure of airway T cell cytoxicity were confounded by a high death rate of both peptide pulsed and un-pulsed cells. It could be argued, however, that monitoring T cell number, cytokine responses and viral load during infection in "real time" gives a more realistic assessment of the relative importance (or lack, thereof) of the cytokine response to viral clearance. Although beyond the scope of this work, such data permit mathematical modelling of the response to influenza infection in the context of pre-existing mucosal T cells – a key step towards establishing the quantitative thresholds at which local memory T cells confer protection without clinical disease for a given pathogen.

To what degree is the enhancement of T cell responses seen with 4-1BB stimulation due to direct T cell stimulation or indirect through transactivation of DC?

Whilst we have demonstrated that 4-1BBL over-expression contributes indirectly to T cell responses through DC activation, we have not quantified this impact relative to direct T cell 4-1BB stimulation. This has important implications for the current use of 4-1BB stimulation for T cell-based therapeutic or prophylactic vaccination strategies. Treating unimmunised mice with anti-4-1BB antibody results in non-specific CD8+ memory T cell expansion but also a number of pathologies such as anaemia,

thrombocytopaenia and substantial liver inflammation[630]. Repeated doses of administration of anti-4-1BB to hepatitis B transgenic mice, for example, substantially worsens liver inflammation and promotes the progression to hepatocarcinoma[632] and a recent review reported that anti-4-1BB agonist therapy for human melanoma resulted in grade IV hepatitis in some individuals[633]. It was recently demonstrated that the latter cannot be entirely mediated by activation of liver-resident memory T cells through 4-1BB, since 4-1BB$^{-/-}$ mice reconstituted with 4-1BB-sufficient memory T cells do not develop hepatic inflammation when stimulated with doses of anti-4-1BB mAb that induce hepatitis in WT mice[631]. 4-1BB expression on both T cells and non-T cells is therefore required to induce immunopathology with anti-4-1BB stimulation.

We propose that non-specific activation of DC by 4-1BB stimulation, as demonstrated in this study, may explain the loss of tolerogenic antigen presentation in organs such as the liver, and subsequent T cell-mediated tissue injury. In order to maximise the efficacy and safety of 4-1BB stimulation for vaccination, the relative contribution of direct and indirect T cell stimulation mediated by this approach needs to be quantified.

One way of achieving this in our vaccination model would be to down-regulate the responsiveness of antigen-bearing DC to 4-1BB stimulation by 4-1BB knockdown. Vaccination of mice with an LV expressing antigen with or without an shRNA against 4-1BB could be combined with anti-4-1BB antibody therapy (or vaccination with an LV expressing 4-1BBL given *in trans* at a separate site draining to the same lymph node). T cell responses in the spleen or lymph node could then be compared. This experiment is now underway.

7.2 LV as mucosal vaccines for lung disease

The delicate architecture of the lung, adapted for gas exchange, is highly susceptible to disruption by inflammation. Homeostatic mechanisms thus exist to ensure a high threshold for inflammation initiation and its suppression after infection clearance. This makes both priming and maintaining a protective T cell memory population in the lung difficult. We have exploited the tropism of LV for AM to develop a SC-IN vaccination regimen that protects against an otherwise lethal challenge with a highly pathogenic influenza strain, without signs of clinical disease. This is achieved through the generation of high numbers of lung and airway NP-specific T cells, summarised in Figure 7-1. We observed rapid viral elimination after vFLIP-NP SC-IN vaccination, without accumulation of pro-inflammatory cytokines or histopathological signs of injury. Contrastingly, both the secondary T cell response seen in subcutaneously vFLIP-NP immunized mice and also the naïve response were characterised by high total cytokine burdens during infection. These levels were greatest when high T cell numbers and viral titres coincide, but low in the absence of either component. Accordingly, a very large resident memory T cell population can achieve such rapid influenza elimination that stimulation of a deleterious secondary effector cytokine response is altogether avoided.

Uniquely amongst mucosal T cell vaccines developed to date (to our knowledge), the protection conferred by these lung-resident T cell populations is sustained for at least 4 months after SC-IN vFLIP-NP vaccination. This suggests significant potential in the clinical domain. We have demonstrated several other attributes of SC-IN vaccination that point towards clinical utility, such as cross-strain protection despite NP sequence variation, boosting of infection-acquired T cell memory and *in vitro* recall of human T cell responses against NP.

At the time of writing, LV are being assessed as therapeutic vaccines in HIV in human trials[66]. However, the clinical potential of LV for prophylactic vaccination against influenza is limited, for now, by the theoretical risks of insertional mutagenesis which outweigh the potential benefits to healthy individuals. Non-integrating LV failed to generate significant lung-based T cell populations and will require substantial

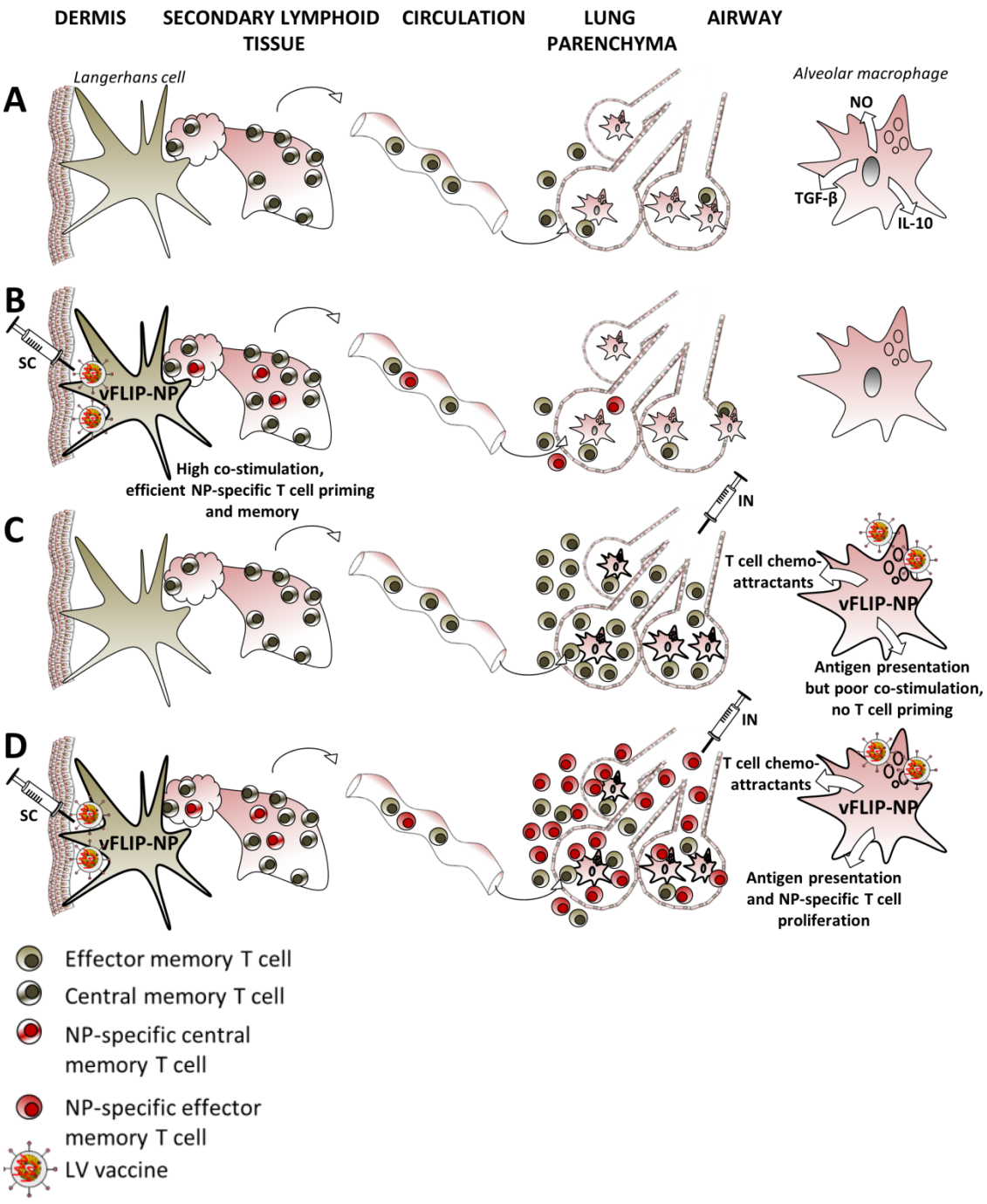

DERMIS
SECONDARY LYMPHOID TISSUE
CIRCULATION
LUNG PARENCHYMA
AIRWAY
A
Langerhans cell
Alveolar macrophage
NO
TGF-β
IL-10
B
SC
vFLIP-NP
High co-stimulation, efficient NP-specific T cell priming and memory
C
IN
T cell chemo-attractants
vFLIP-NP
Antigen presentation but poor co-stimulation, no T cell priming
D
SC
vFLIP-NP
IN
T cell chemo-attractants
vFLIP-NP
Antigen presentation and NP-specific T cell proliferation
Effector memory T cell
Central memory T cell
NP-specific central memory T cell
NP-specific effector memory T cell
LV vaccine

Figure 7-1 LV vaccination routes and generation of mucosal T cell memory populations. **(A)** In the steady state, central memory T cells supply a circulating effector memory population that migrates into lung parenchyma and the airway. Numbers of airway T cells are very low and AM maintain an high threshold for local priming of naïve T cells. Sub-lethal Influenza challenge will iniate a slow primary effector T cell response which takes 6-9 days to generate significant lung and airway influenza-specific T cell populations. **(B)** SC vaccination with LV vFLIP-NP transduces Langerhans cells which migrate to lymph nodes and prime and expand NP-specific primary effector T cells. NP-specific central and effector memory T cells are later proportionately represented in the lung and airway but here T cell numbers are equivalent to those in the naïve mouse. Lethal influenza challenge results in recruitment and expansion of circulating NP-specific effector memory T cells which approach peak numbers between day 3 and 6, mediating viral clearance and recovery in the majority of vaccinated mice but not without substantial lung injury. **(C)** IN LV vaccination with vFLIP-NP results in secretion of T cell chemoattractants by large numbers of transduced AM. Expression of co-stimulation molecules by AM is minimal, however, and IN vFLIP-NP fails to prime a detectable NP-specific T cell response. Netiher non-specific recruitment of circulating effector memory T cells to the lung nor NFκB activation of AM has any impact on the clinical course of lethal influenza challenge. **(D)** IN vFLIP-NP vaccination in mice subcutaneoulsy primed with the same vaccine (or with NP-specific T cell memory established by prior infection) recruits large numbers of NP-specific circulating effector memory T cells to the lung and airway, therein enriched through antigen-driven proliferation by presentation of NP epitopes by large numbers of LV-transduced AM. Lethal influenza challenge is rapidly cleared by the large, sustained tissue-resident population of NP-specific effector memory T cells, before an injurious secondary effector T cell cytokine response is initiated.

improvement of levels of expression and persistence of transgenes in order to establish and maintain protective lung-based T cell populations.

However, SC-IN LV vaccination may have applications in other lung diseases where the cost and risk limitations of prophylactic vaccination in healthy individuals do not apply. Inoperable lung cancer and extensively drug resistant (XDR) TB have similarly poor prognoses and both are prime candidates for T cell-based therapeutic strategies. We are now testing the efficacy of SC-IN LV vaccination as a means of generating T cell responses against the TB antigen Ag85 and against epidermal growth factor receptor (EGFR) in a murine lung cancer model.

8 References

1. Roush SW, M. T. HIstorical comparisons of morbidity and mortality for vaccine-preventable diseases in the united states. *JAMA J. Am. Med. Assoc.* **298,** 2155–2163 (2007).
2. WHO | Influenza (Seasonal). at http://www.who.int/mediacentre/factsheets/fs211/en/>
3. He, X.-S. *et al.* Cellular Immune Responses in Children and Adults Receiving Inactivated or Live Attenuated Influenza Vaccines. *J. Virol.* **80,** 11756–11766 (2006).
4. Webster, R. G. & Askonas, B. A. Cross-protection and cross-reactive cytotoxic T cells induced by influenza virus vaccines in mice. *Eur. J. Immunol.* **10,** 396–401 (1980).
5. Bodewes, R. *et al.* Annual influenza vaccination affects the development of heterosubtypic immunity. *Vaccine* **30,** 7407–7410 (2012).
6. Bodewes, R., Kreijtz, J. H. & Rimmelzwaan, G. F. Yearly influenza vaccinations: a double-edged sword? *Lancet Infect. Dis.* **9,** 784–788 (2009).
7. Kapikian, A. Z., Mitchell, R. H., Chanock, R. M., Shvedoff, R. A. & Stewart, C. E. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am. J. Epidemiol.* **89,** 405–421 (1969).
8. Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179,** 1109–1118 (1994).
9. Munn, D. H. *et al.* Potential Regulatory Function of Human Dendritic Cells Expressing Indoleamine 2,3-Dioxygenase. *Science* **297,** 1867–1870 (2002).
10. Palucka, K. & Banchereau, J. Cancer immunotherapy via dendritic cells. *Nat. Rev. Cancer* **12,** 265–277 (2012).
11. Higano, C. S. *et al.* Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer. *Cancer* **115,** 3670–3679 (2009).
12. Rinaldo, C. R. Dendritic cell-based human immunodeficiency virus vaccine. *J. Intern. Med.* **265,** 138–158 (2009).
13. Strategies for Management of Antiretroviral Therapy (SMART) Study Group *et al.* CD4+ count-guided interruption of antiretroviral treatment. *N. Engl. J. Med.* **355,** 2283–2296 (2006).
14. Konduri, V., Decker, W. K., Halpert, M. M., Gilbert, B. & Safdar, A. Modeling dendritic cell vaccination for influenza prophylaxis: potential applications for niche populations. *J. Infect. Dis.* **207,** 1764–1772 (2013).

15. Gringhuis, S. I. *et al.* C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity* **26,** 605–616 (2007).
16. Boruchov, A. M. *et al.* Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. *J. Clin. Invest.* **115,** 2914–2923 (2005).
17. Guyre, P. M. *et al.* Increased potency of Fc-receptor-targeted antigens. *Cancer Immunol. Immunother. CII* **45,** 146–148 (1997).
18. Loureiro, S. *et al.* Adjuvant-Free Immunization with Hemagglutinin-Fc Fusion Proteins as an Approach to Influenza Vaccines. *J. Virol.* **85,** 3010–3014 (2011).
19. Abdel-Motal, U. M., Guay, H. M., Wigglesworth, K., Welsh, R. M. & Galili, U. Immunogenicity of Influenza Virus Vaccine Is Increased by Anti-Gal-Mediated Targeting to Antigen-Presenting Cells. *J. Virol.* **81,** 9131–9141 (2007).
20. Caminschi, I., Maraskovsky, E. & Heath, W. R. Targeting Dendritic Cells in vivo for Cancer Therapy. *Front. Immunol.* **3,** 13 (2012).
21. Bonifaz, L. C. *et al.* In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J. Exp. Med.* **199,** 815–824 (2004).
22. Pereira, C. F. *et al.* In vivo targeting of DC-SIGN-positive antigen-presenting cells in a nonhuman primate model. *J. Immunother. Hagerstown Md 1997* **30,** 705–714 (2007).
23. Flynn, B. J. *et al.* Immunization with HIV Gag targeted to dendritic cells followed by recombinant New York vaccinia virus induces robust T-cell immunity in nonhuman primates. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 7131–7136 (2011).
24. Flamar, A.-L. *et al.* Noncovalent assembly of anti-dendritic cell antibodies and antigens for evoking immune responses in vitro and in vivo. *J. Immunol. Baltim. Md 1950* **189,** 2645–2655 (2012).
25. Moghimi, S. M. *et al.* Particulate systems for targeting of macrophages: basic and therapeutic concepts. *J. Innate Immun.* **4,** 509–528 (2012).
26. Oxman, M. N. *et al.* A vaccine to prevent herpes zoster and postherpetic neuralgia in older adults. *N. Engl. J. Med.* **352,** 2271–2284 (2005).
27. Powell, T. J. *et al.* Priming with Cold-Adapted Influenza A Does Not Prevent Infection but Elicits Long-Lived Protection against Supralethal Challenge with Heterosubtypic Virus. *J. Immunol.* **178,** 1030–1038 (2007).
28. He, X.-S. *et al.* Cellular Immune Responses in Children and Adults Receiving Inactivated or Live Attenuated Influenza Vaccines. *J. Virol.* **80,** 11756–11766 (2006).
29. Carter, N. J. & Curran, M. P. Live attenuated influenza vaccine (FluMist®; Fluenz™): a review of its use in the prevention of seasonal influenza in children and adults. *Drugs* **71,** 1591–1622 (2011).
30. Fleming, D. M. *et al.* Comparison of the efficacy and safety of live attenuated cold-adapted influenza vaccine, trivalent, with trivalent inactivated influenza virus vaccine in children and adolescents with asthma. *Pediatr. Infect. Dis. J.* **25,** 860–869 (2006).

31. Wolff, J. A. *et al.* Direct gene transfer into mouse muscle in vivo. *Science* **247,** 1465–1468 (1990).
32. Tang, D. C., DeVit, M. & Johnston, S. A. Genetic immunization is a simple method for eliciting an immune response. *Nature* **356,** 152–154 (1992).
33. Ulmer, J. B. *et al.* Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259,** 1745–1749 (1993).
34. Babiuk, S. *et al.* TLR9-/- and TLR9+/+ mice display similar immune responses to a DNA vaccine. *Immunology* **113,** 114–120 (2004).
35. Ishii, K. J. *et al.* TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* **451,** 725–729 (2008).
36. Kwissa, M. *et al.* Efficient vaccination by intradermal or intramuscular inoculation of plasmid DNA expressing hepatitis B surface antigen under desmin promoter/enhancer control. *Vaccine* **18,** 2337–2344 (2000).
37. Laddy, D. J. *et al.* Immunogenicity of novel consensus-based DNA vaccines against avian influenza. *Vaccine* **25,** 2984–2989 (2007).
38. Laddy, D. J. *et al.* Heterosubtypic protection against pathogenic human and avian influenza viruses via in vivo electroporation of synthetic consensus DNA antigens. *PloS One* **3,** e2517 (2008).
39. Mincheff, M. *et al.* Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: a phase I/II clinical trial. *Eur. Urol.* **38,** 208–217 (2000).
40. Yuan, J. *et al.* Safety and immunogenicity of a human and mouse gp100 DNA vaccine in a phase I trial of patients with melanoma. *Cancer Immun.* **9,** 5 (2009).
41. Norell, H. *et al.* Vaccination with a plasmid DNA encoding HER-2/neu together with low doses of GM-CSF and IL-2 in patients with metastatic breast carcinoma: a pilot clinical trial. *J. Transl. Med.* **8,** 53 (2010).
42. Peters, B. S. *et al.* Studies of a prophylactic HIV-1 vaccine candidate based on modified vaccinia virus Ankara (MVA) with and without DNA priming: effects of dosage and route on safety and immunogenicity. *Vaccine* **25,** 2120–2127 (2007).
43. Le, T. P. *et al.* Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. *Vaccine* **18,** 1893–1901 (2000).
44. Tacket, C. O. *et al.* Phase 1 safety and immune response studies of a DNA vaccine encoding hepatitis B surface antigen delivered by a gene delivery device. *Vaccine* **17,** 2826–2829 (1999).
45. Kutzler, M. A. & Weiner, D. B. DNA vaccines: ready for prime time? *Nat. Rev. Genet.* **9,** 776–788 (2008).
46. Rovere, P. *et al.* Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. *J. Immunol. Baltim. Md 1950* **161,** 4467–4471 (1998).
47. Ulmer, J. B. *et al.* Expression of a viral protein by muscle cells in vivo induces protective cell-mediated immunity. *Vaccine* **15,** 839–841 (1997).

48. Kurts, C., Heath, W. R., Kosaka, H., Miller, J. F. & Carbone, F. R. The peripheral deletion of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). *J. Exp. Med.* **188,** 415–420 (1998).
49. Kim, J. J. *et al.* Macrophage colony-stimulating factor can modulate immune responses and attract dendritic cells in vivo. *Hum. Gene Ther.* **11,** 305–321 (2000).
50. Sin, J. I. *et al.* Protective immunity against heterologous challenge with encephalomyocarditis virus by VP1 DNA vaccination: effect of coinjection with a granulocyte-macrophage colony stimulating factor gene. *Vaccine* **15,** 1827–1833 (1997).
51. Geissler, M., Gesien, A., Tokushige, K. & Wands, J. R. Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. *J. Immunol. Baltim. Md 1950* **158,** 1231–1237 (1997).
52. Fuller, D. H., Loudon, P. & Schmaljohn, C. Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. *Methods San Diego Calif* **40,** 86–97 (2006).
53. Murakami, T. & Sunada, Y. Plasmid DNA gene therapy by electroporation: principles and recent advances. *Curr. Gene Ther.* **11,** 447–456 (2011).
54. Dai, B. *et al.* HIV-1 Gag-specific immunity induced by a lentivector-based vaccine directed to dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 20382–20387 (2009).
55. Nathwani, A. C. *et al.* Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N. Engl. J. Med.* **365,** 2357–2365 (2011).
56. Nathwani, A. C. *et al.* Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* **107,** 2653–2661 (2006).
57. Rerks-Ngarm, S. *et al.* Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* **361,** 2209–2220 (2009).
58. Sheehy, S. H. *et al.* ChAd63-MVA-vectored Blood-stage Malaria Vaccines Targeting MSP1 and AMA1: Assessment of Efficacy Against Mosquito Bite Challenge in Humans. *Mol. Ther. J. Am. Soc. Gene Ther.* **20,** 2355–2368 (2012).
59. Zhi, Y. *et al.* Efficacy of severe acute respiratory syndrome vaccine based on a nonhuman primate adenovirus in the presence of immunity against human adenovirus. *Hum. Gene Ther.* **17,** 500–506 (2006).
60. Sullivan, N. J. *et al.* Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* **424,** 681–684 (2003).
61. Van Kampen, K. R. *et al.* Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans. *Vaccine* **23,** 1029–1036 (2005).
62. Ockenhouse, C. F. *et al.* Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for Plasmodium falciparum malaria. *J. Infect. Dis.* **177,** 1664–1673 (1998).

63. Mwau, M. *et al.* A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. *J. Gen. Virol.* **85,** 911–919 (2004).
64. Vardas, E. *et al.* A phase 2 study to evaluate the safety and immunogenicity of a recombinant HIV type 1 vaccine based on adeno-associated virus. *AIDS Res. Hum. Retroviruses* **26,** 933–942 (2010).
65. Todo, T. Active immunotherapy: oncolytic virus therapy using HSV-1. *Adv. Exp. Med. Biol.* **746,** 178–186 (2012).
66. Rodriguez, M. *et al.* Design and development of a new lentiviral based anti-HIV therapeutic vaccine. *Retrovirology* **9,** P350 (2012).
67. Tuosto, L. & Acuto, O. CD28 affects the earliest signaling events generated by TCR engagement. *Eur. J. Immunol.* **28,** 2131–2142 (1998).
68. Kopf, M. *et al.* OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL Responses after virus infection. *Immunity* **11,** 699–708 (1999).
69. Gramaglia, I., Weinberg, A. D., Lemon, M. & Croft, M. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J. Immunol. Baltim. Md 1950* **161,** 6510–6517 (1998).
70. Whitmire, J. K. *et al.* CD40-CD40 ligand costimulation is required for generating antiviral CD4 T cell responses but is dispensable for CD8 T cell responses. *J. Immunol. Baltim. Md 1950* **163,** 3194–3201 (1999).
71. Whitmire, J. K. *et al.* CD40-CD40 ligand costimulation is required for generating antiviral CD4 T cell responses but is dispensable for CD8 T cell responses. *J. Immunol. Baltim. Md 1950* **163,** 3194–3201 (1999).
72. Shahinian, A. *et al.* Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261,** 609–612 (1993).
73. Tan, J. T., Whitmire, J. K., Ahmed, R., Pearson, T. C. & Larsen, C. P. 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses. *J. Immunol. Baltim. Md 1950* **163,** 4859–4868 (1999).
74. Borrow, P. *et al.* CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. *J. Exp. Med.* **183,** 2129–2142 (1996).
75. Lin, G. H. Y. *et al.* Endogenous 4-1BB ligand plays a critical role in protection from influenza-induced disease. *J. Immunol. Baltim. Md 1950* **182,** 934–947 (2009).
76. Schmidt, C. S. & Mescher, M. F. Adjuvant effect of IL-12: conversion of peptide antigen administration from tolerizing to immunizing for CD8+ T cells in vivo. *J. Immunol. Baltim. Md 1950* **163,** 2561–2567 (1999).
77. Ben-Sasson, S. Z. *et al.* IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 7119–7124 (2009).

78. Salio, M., Cella, M., Suter, M. & Lanzavecchia, A. Inhibition of dendritic cell maturation by herpes simplex virus. *Eur. J. Immunol.* **29,** 3245–3253 (1999).
79. Kruse, M. *et al.* Mature dendritic cells infected with herpes simplex virus type 1 exhibit inhibited T-cell stimulatory capacity. *J. Virol.* **74,** 7127–7136 (2000).
80. Jenne, L., Hauser, C., Arrighi, J.-F., Saurat, J.-H. & Hügin, A. W. Poxvirus as a vector to transduce human dendritic cells for immunotherapy: Abortive infection but reduced APC function. *Gene Ther.* **7,** 1575–1583 (2000).
81. Drillien, R., Spehner, D., Bohbot, A. & Hanau, D. Vaccinia virus-related events and phenotypic changes after infection of dendritic cells derived from human monocytes. *Virology* **268,** 471–481 (2000).
82. Kaczmarek, A., Vandenabeele, P. & Krysko, D. V. Necroptosis: The Release of Damage-Associated Molecular Patterns and Its Physiological Relevance. *Immunity* **38,** 209–223 (2013).
83. Assarsson, E. *et al.* Immunomic analysis of the repertoire of T-cell specificities for influenza A virus in humans. *J. Virol.* **82,** 12241–12251 (2008).
84. Lee, L. Y.-H. *et al.* Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *J. Clin. Invest.* **118,** 3478–3490 (2008).
85. Kim, T. S. & Braciale, T. J. Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic CD8+ T cell responses. *PLoS ONE* **4,** e4204 (2009).
86. Hao, X., Kim, T. S. & Braciale, T. J. Differential response of respiratory dendritic cell subsets to influenza virus infection. *J. Virol.* **82,** 4908–4919 (2008).
87. Belz, G. T., Stevenson, P. G. & Doherty, P. C. Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses. *J. Immunol. Baltim. Md 1950* **165,** 2404–2409 (2000).
88. Yewdell, J. W. & Bennink, J. R. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu. Rev. Immunol.* **17,** 51–88 (1999).
89. Deng, Y., Yewdell, J. W., Eisenlohr, L. C. & Bennink, J. R. MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL. *J. Immunol. Baltim. Md 1950* **158,** 1507–1515 (1997).
90. Mempel, T. R., Henrickson, S. E. & Von Andrian, U. H. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* **427,** 154–159 (2004).
91. Iezzi, G., Karjalainen, K. & Lanzavecchia, A. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* **8,** 89–95 (1998).
92. Iezzi, G., Scotet, E., Scheidegger, D. & Lanzavecchia, A. The interplay between the duration of TCR and cytokine signaling determines T cell polarization. *Eur. J. Immunol.* **29,** 4092–4101 (1999).

93. Obst, R., van Santen, H.-M., Mathis, D. & Benoist, C. Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. *J. Exp. Med.* **201,** 1555–1565 (2005).
94. Van Stipdonk, M. J., Lemmens, E. E. & Schoenberger, S. P. Naïve CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat. Immunol.* **2,** 423–429 (2001).
95. Mercado, R. *et al.* Early programming of T cell populations responding to bacterial infection. *J. Immunol. Baltim. Md 1950* **165,** 6833–6839 (2000).
96. Kaech, S. M. & Ahmed, R. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nat. Immunol.* **2,** 415–422 (2001).
97. Wherry, E. J., Puorro, K. A., Porgador, A. & Eisenlohr, L. C. The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. *J. Immunol. Baltim. Md 1950* **163,** 3735–3745 (1999).
98. Van Heijst, J. W. J. *et al.* Recruitment of antigen-specific CD8+ T cells in response to infection is markedly efficient. *Science* **325,** 1265–1269 (2009).
99. Plesa, G., McKenna, P. M., Schnell, M. J. & Eisenlohr, L. C. Immunogenicity of Cytopathic and Noncytopathic Viral Vectors. *J. Virol.* **80,** 6259–6266 (2006).
100. Lasaro, M. O. & Ertl, H. C. J. New insights on adenovirus as vaccine vectors. *Mol. Ther.* **17,** 1333–1339 (2009).
101. Benlahrech, A. *et al.* Adenovirus vector vaccination induces expansion of memory CD4 T cells with a mucosal homing phenotype that are readily susceptible to HIV-1. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 19940–19945 (2009).
102. Gudmundsdotter, L. *et al.* Recombinant Modified Vaccinia Ankara (MVA) effectively boosts DNA-primed HIV-specific immune responses in humans despite pre-existing vaccinia immunity. *Vaccine* **27,** 4468–4474 (2009).
103. Asefa, B., Korokhov, N. & Lemiale, F. Heterologous HIV-based lentiviral/adenoviral vectors immunizations result in enhanced HIV-specific immunity. *Vaccine* **28,** 3617–3624 (2010).
104. Schneider, J. *et al.* Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat. Med.* **4,** 397–402 (1998).
105. Rerks-Ngarm, S. *et al.* Vaccination with ALVAC and AIDSVAX to Prevent HIV-1 Infection in Thailand. *N. Engl. J. Med.* **361,** 2209–2220 (2009).
106. Mare, A. de *et al.* Viral vector-based prime-boost immunization regimens: a possible involvement of T-cell competition. *Gene Ther.* **15,** 393–403 (2008).
107. Zufferey, R. *et al.* Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72,** 9873–9880 (1998).
108. Buffa, V. *et al.* A single administration of lentiviral vectors expressing either full-length human immunodeficiency virus 1 (HIV-1)(HXB2) Rev/Env or codon-

optimized HIV-1(JR-FL) gp120 generates durable immune responses in mice. *J. Gen. Virol.* **87,** 1625–1634 (2006).

109. Dai, B. *et al.* HIV-1 Gag-specific immunity induced by a lentivector-based vaccine directed to dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 20382–20387 (2009).
110. Asefa, B., Korokhov, N. & Lemiale, F. Heterologous HIV-based lentiviral/adenoviral vectors immunizations result in enhanced HIV-specific immunity. *Vaccine* **28,** 3617–3624 (2010).
111. Lemiale, F. *et al.* An HIV-based lentiviral vector as HIV vaccine candidate: Immunogenic characterization. *Vaccine* **28,** 1952–1961 (2010).
112. Wu, L. & KewalRamani, V. N. Dendritic-cell interactions with HIV: infection and viral dissemination. *Nat. Rev. Immunol.* **6,** 859–868 (2006).
113. Blanchet, F. P. *et al.* Human Immunodeficiency Virus-1 Inhibition of Immunoamphisomes in Dendritic Cells Impairs Early Innate and Adaptive Immune Responses. *Immunity* **32,** 654–669 (2010).
114. Esslinger, C. *et al.* In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses. *J. Clin. Invest.* **111,** 1673–1681 (2003).
115. Palmowski, M. J. *et al.* Intravenous injection of a lentiviral vector encoding NY-ESO-1 induces an effective CTL response. *J. Immunol. Baltim. Md 1950* **172,** 1582–1587 (2004).
116. Kim, J. H. *et al.* Induction of therapeutic antitumor immunity by in vivo administration of a lentiviral vaccine. *Hum. Gene Ther.* **16,** 1255–1266 (2005).
117. Chapatte, L., Colombetti, S., Cerottini, J.-C. & Lévy, F. Efficient induction of tumor antigen-specific CD8+ memory T cells by recombinant lentivectors. *Cancer Res.* **66,** 1155–1160 (2006).
118. Kimura, T. *et al.* Lentiviral vectors with CMV or MHCII promoters administered in vivo: immune reactivity versus persistence of expression. *Mol. Ther. J. Am. Soc. Gene Ther.* **15,** 1390–1399 (2007).
119. Garcia Casado, J. *et al.* Lentivector immunization induces tumor antigen-specific B and T cell responses in vivo. *Eur. J. Immunol.* **38,** 1867–1876 (2008).
120. Lopes, L. *et al.* Immunization with a lentivector that targets tumor antigen expression to dendritic cells induces potent CD8+ and CD4+ T-cell responses. *J. Virol.* **82,** 86–95 (2008).
121. Liu, Y. *et al.* Lentivector immunization stimulates potent CD8 T cell responses against melanoma self-antigen tyrosinase-related protein 1 and generates antitumor immunity in mice. *J. Immunol. Baltim. Md 1950* **182,** 5960–5969 (2009).
122. Dullaers, M. *et al.* Induction of effective therapeutic antitumor immunity by direct in vivo administration of lentiviral vectors. *Gene Ther.* **13,** 630–640 (2006).
123. He, Y., Zhang, J., Mi, Z., Robbins, P. & Falo, L. D., Jr. Immunization with lentiviral vector-transduced dendritic cells induces strong and long-lasting T cell responses and therapeutic immunity. *J. Immunol. Baltim. Md 1950* **174,** 3808–3817 (2005).

124. Rowe, H. M. *et al.* Immunization with a lentiviral vector stimulates both CD4 and CD8 T cell responses to an ovalbumin transgene. *Mol. Ther. J. Am. Soc. Gene Ther.* **13,** 310–319 (2006).
125. Rowe, H. M. *et al.* Expression of vFLIP in a lentiviral vaccine vector activates NF-{kappa}B, matures dendritic cells, and increases CD8+ T-cell responses. *J. Virol.* **83,** 1555–1562 (2009).
126. Karwacz, K. *et al.* Nonintegrating Lentivector Vaccines Stimulate Prolonged T-Cell and Antibody Responses and Are Effective in Tumor Therapy. *J. Virol.* **83,** 3094–3103 (2009).
127. Escors, D. *et al.* Targeting dendritic cell signaling to regulate the response to immunization. *Blood* **111,** 3050–3061 (2008).
128. Loisel-Meyer, S. *et al.* Potent induction of B- and T-cell immunity against human carcinoembryonic antigen-expressing tumors in human carcinoembryonic antigen transgenic mice mediated by direct lentivector injection. *Mol. Cancer Ther.* **8,** 692–702 (2009).
129. Grasso, F. *et al.* Successful therapeutic vaccination with integrase defective lentiviral vector expressing nononcogenic human papillomavirus E7 protein. *Int. J. Cancer J. Int. Cancer* **132,** 335–344 (2013).
130. Iglesias, M. C. *et al.* A single immunization with a minute dose of a lentiviral vector-based vaccine is highly effective at eliciting protective humoral immunity against West Nile virus. *J. Gene Med.* **8,** 265–274 (2006).
131. Negri, D. R. M. *et al.* Transduction of human antigen-presenting cells with integrase-defective lentiviral vector enables functional expansion of primed antigen-specific CD8(+) T cells. *Hum. Gene Ther.* **21,** 1029–1035 (2010).
132. Chiuppesi, F. *et al.* A lentiviral vector-based, herpes simplex virus 1 (HSV-1) glycoprotein B vaccine affords cross-protection against HSV-1 and HSV-2 genital infections. *J. Virol.* **86,** 6563–6574 (2012).
133. Iglesias, M. C. *et al.* Lentiviral vectors encoding HIV-1 polyepitopes induce broad CTL responses in vivo. *Mol. Ther. J. Am. Soc. Gene Ther.* **15,** 1203–1210 (2007).
134. Negri, D. R. M. *et al.* Successful immunization with a single injection of non-integrating lentiviral vector. *Mol. Ther. J. Am. Soc. Gene Ther.* **15,** 1716–1723 (2007).
135. Buffa, V. *et al.* A single administration of lentiviral vectors expressing either full-length human immunodeficiency virus 1 (HIV-1)(HXB2) Rev/Env or codon-optimized HIV-1(JR-FL) gp120 generates durable immune responses in mice. *J. Gen. Virol.* **87,** 1625–1634 (2006).
136. Beignon, A.-S. *et al.* Lentiviral vector-based prime/boost vaccination against AIDS: pilot study shows protection against Simian immunodeficiency virus SIVmac251 challenge in macaques. *J. Virol.* **83,** 10963–10974 (2009).
137. Esslinger, C., Romero, P. & MacDonald, H. R. Efficient transduction of dendritic cells and induction of a T-cell response by third-generation lentivectors. *Hum. Gene Ther.* **13,** 1091–1100 (2002).

138. Rouas, R. *et al.* Lentiviral-mediated gene delivery in human monocyte-derived dendritic cells: optimized design and procedures for highly efficient transduction compatible with clinical constraints. *Cancer Gene Ther.* **9,** 715–724 (2002).
139. Schroers, R. *et al.* Transduction of human PBMC-derived dendritic cells and macrophages by an HIV-1-based lentiviral vector system. *Mol. Ther. J. Am. Soc. Gene Ther.* **1,** 171–179 (2000).
140. Chinnasamy, N. *et al.* Efficient gene transfer to human peripheral blood monocyte-derived dendritic cells using human immunodeficiency virus type 1-based lentiviral vectors. *Hum. Gene Ther.* **11,** 1901–1909 (2000).
141. Pincha, M., Sundarasetty, B. S. & Stripecke, R. Lentiviral vectors for immunization: an inflammatory field. *Expert Rev. Vaccines* **9,** 309–321 (2010).
142. Zarei, S., Leuba, F., Arrighi, J.-F., Hauser, C. & Piguet, V. Transduction of dendritic cells by antigen-encoding lentiviral vectors permits antigen processing and MHC class I-dependent presentation. *J. Allergy Clin. Immunol.* **109,** 988–994 (2002).
143. He, Y., Zhang, J., Donahue, C. & Falo, L. D., Jr. Skin-derived dendritic cells induce potent CD8(+) T cell immunity in recombinant lentivector-mediated genetic immunization. *Immunity* **24,** 643–656 (2006).
144. Esslinger, C., Romero, P. & MacDonald, H. R. Efficient transduction of dendritic cells and induction of a T-cell response by third-generation lentivectors. *Hum. Gene Ther.* **13,** 1091–1100 (2002).
145. He, Y., Zhang, J., Donahue, C. & Falo, L. D., Jr. Skin-derived dendritic cells induce potent CD8(+) T cell immunity in recombinant lentivector-mediated genetic immunization. *Immunity* **24,** 643–656 (2006).
146. Arce, F., Rowe, H. M., Chain, B., Lopes, L. & Collins, M. K. Lentiviral vectors transduce proliferating dendritic cell precursors leading to persistent antigen presentation and immunization. *Mol. Ther. J. Am. Soc. Gene Ther.* **17,** 1643–1650 (2009).
147. Humbert, J.-M. *et al.* Measles Virus Glycoprotein-Pseudotyped Lentiviral Vectors Are Highly Superior to Vesicular Stomatitis Virus G Pseudotypes for Genetic Modification of Monocyte-Derived Dendritic Cells. *J. Virol.* **86,** 5192–5203 (2012).
148. Lahouassa, H. *et al.* SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates. *Nat. Immunol.* **13,** 223–228 (2012).
149. Durand, S. *et al.* Tailored HIV-1 Vectors for Genetic Modification of Primary Human Dendritic Cells and Monocytes. *J. Virol.* **87,** 234–242 (2013).
150. Annoni, A. *et al.* In vivo delivery of a microRNA-regulated transgene induces antigen-specific regulatory T cells and promotes immunologic tolerance. *Blood* **114,** 5152–5161 (2009).
151. Ageichik, A., Buchholz, C. J. & Collins, M. K. Lentiviral vectors targeted to MHC II are effective in immunization. *Hum. Gene Ther.* **22,** 1249–1254 (2011).
152. Breckpot, K. *et al.* HIV-1 lentiviral vector immunogenicity is mediated by Toll-like receptor 3 (TLR3) and TLR7. *J. Virol.* **84,** 5627–5636 (2010).

153. Breckpot, K. *et al.* Activation of immature monocyte-derived dendritic cells after transduction with high doses of lentiviral vectors. *Hum. Gene Ther.* **18,** 536–546 (2007).
154. Koya, R. C. *et al.* Potent maturation of monocyte-derived dendritic cells after CD40L lentiviral gene delivery. *J. Immunother. Hagerstown Md 1997* **26,** 451–460 (2003).
155. Kim, J. H. *et al.* Induction of therapeutic antitumor immunity by in vivo administration of a lentiviral vaccine. *Hum. Gene Ther.* **16,** 1255–1266 (2005).
156. Akazawa, T. *et al.* Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 252–257 (2007).
157. Rowe, H. M. *et al.* Expression of vFLIP in a lentiviral vaccine vector activates NF-{kappa}B, matures dendritic cells, and increases CD8+ T-cell responses. *J. Virol.* **83,** 1555–1562 (2009).
158. Karwacz, K. *et al.* Nonintegrating lentivector vaccines stimulate prolonged T-cell and antibody responses and are effective in tumor therapy. *J. Virol.* **83,** 3094–3103 (2009).
159. Breckpot, K. *et al.* Attenuated expression of A20 markedly increases the efficacy of double-stranded RNA-activated dendritic cells as an anti-cancer vaccine. *J. Immunol. Baltim. Md 1950* **182,** 860–870 (2009).
160. Karwacz, K. *et al.* PD-L1 co-stimulation contributes to ligand-induced T cell receptor down-modulation on CD8+ T cells. *EMBO Mol. Med.* **3,** 581–592 (2011).
161. VandenDriessche, T. *et al.* Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. *Blood* **100,** 813–822 (2002).
162. Arce, F., Rowe, H. M., Chain, B., Lopes, L. & Collins, M. K. Lentiviral Vectors Transduce Proliferating Dendritic Cell Precursors Leading to Persistent Antigen Presentation and Immunization. *Mol. Ther. J. Am. Soc. Gene Ther.* **17,** 1643–1650 (2009).
163. Asefa, B., Korokhov, N. & Lemiale, F. Heterologous HIV-based lentiviral/adenoviral vectors immunizations result in enhanced HIV-specific immunity. *Vaccine* **28,** 3617–3624 (2010).
164. Li, G.-B. & Lu, G.-X. Gene delivery efficiency in bone marrow-derived dendritic cells: comparison of four methods and optimization for lentivirus transduction. *Mol. Biotechnol.* **43,** 250–256 (2009).
165. Croyle, M. A. *et al.* PEGylation of a Vesicular Stomatitis Virus G Pseudotyped Lentivirus Vector Prevents Inactivation in Serum. *J. Virol.* **78,** 912–921 (2004).
166. Beignon, A.-S. *et al.* Lentiviral Vector-Based Prime/Boost Vaccination against AIDS: Pilot Study Shows Protection against Simian Immunodeficiency Virus SIVmac251 Challenge in Macaques. *J. Virol.* **83,** 10963–10974 (2009).

167. Asefa, B., Korokhov, N. & Lemiale, F. Heterologous HIV-based lentiviral/adenoviral vectors immunizations result in enhanced HIV-specific immunity. *Vaccine* **28,** 3617–3624 (2010).
168. Miao, H. *et al.* Quantifying the Early Immune Response and Adaptive Immune Response Kinetics in Mice Infected with Influenza A Virus. *J. Virol.* **84,** 6687–6698 (2010).
169. LaMere, M. W. *et al.* Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. *J. Immunol. Baltim. Md 1950* **186,** 4331–4339 (2011).
170. Skehel, J. J. & Wiley, D. C. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem.* **69,** 531–569 (2000).
171. Pinto, L. H. & Lamb, R. A. The M2 proton channels of influenza A and B viruses. *J. Biol. Chem.* **281,** 8997–9000 (2006).
172. Boulo, S., Akarsu, H., Ruigrok, R. W. H. & Baudin, F. Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. *Virus Res.* **124,** 12–21 (2007).
173. Crow, M., Deng, T., Addley, M. & Brownlee, G. G. Mutational analysis of the influenza virus cRNA promoter and identification of nucleotides critical for replication. *J. Virol.* **78,** 6263–6270 (2004).
174. Dhar, R., Chanock, R. M. & Lai, C. J. Nonviral oligonucleotides at the 5' terminus of cytoplasmic influenza viral mRNA deduced from cloned complete genomic sequences. *Cell* **21,** 495–500 (1980).
175. Engelhardt, O. G. & Fodor, E. Functional association between viral and cellular transcription during influenza virus infection. *Rev. Med. Virol.* **16,** 329–345 (2006).
176. Fortes, P., Lamond, A. I. & Ortín, J. Influenza virus NS1 protein alters the subnuclear localization of cellular splicing components. *J. Gen. Virol.* **76 (Pt 4),** 1001–1007 (1995).
177. Shimizu, K., Iguchi, A., Gomyou, R. & Ono, Y. Influenza virus inhibits cleavage of the HSP70 pre-mRNAs at the polyadenylation site. *Virology* **254,** 213–219 (1999).
178. Poon, L. L., Pritlove, D. C., Fodor, E. & Brownlee, G. G. Direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template. *J. Virol.* **73,** 3473–3476 (1999).
179. CAIRNS, H. J. F., EDNEY, M. & FAZEKAS DE ST GROTH, S. Quantitative aspects of influenza virus multiplication. *J. Immunol. Baltim. Md 1950* **69,** 155–181 (1952).
180. Stray, S. J. & Air, G. M. Apoptosis by influenza viruses correlates with efficiency of viral mRNA synthesis. *Virus Res.* **77,** 3–17 (2001).
181. Boulo, S., Akarsu, H., Ruigrok, R. W. H. & Baudin, F. Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. *Virus Res.* **124,** 12–21 (2007).
182. Iwatsuki-Horimoto, K. *et al.* The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. *J. Virol.* **80,** 5233–5240 (2006).
183. Enami, M., Sharma, G., Benham, C. & Palese, P. An influenza virus containing nine different RNA segments. *Virology* **185,** 291–298 (1991).

184. Fujii, Y., Goto, H., Watanabe, T., Yoshida, T. & Kawaoka, Y. Selective incorporation of influenza virus RNA segments into virions. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 2002–2007 (2003).
185. Fujii, K. *et al.* Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. *J. Virol.* **79,** 3766–3774 (2005).
186. Rabadan, R., Levine, A. J. & Krasnitz, M. Non-random reassortment in human influenza A viruses. *Influenza Other Respir. Viruses* **2,** 9–22 (2008).
187. Nayak, D. P., Hui, E. K.-W. & Barman, S. Assembly and budding of influenza virus. *Virus Res.* **106,** 147–165 (2004).
188. Palese, P., Tobita, K., Ueda, M. & Compans, R. W. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology* **61,** 397–410 (1974).
189. Julkunen, I. *et al.* Inflammatory responses in influenza A virus infection. *Vaccine* **19, Supplement 1,** S32–S37 (2000).
190. Santoli, D., Trinchieri, G. & Koprowski, H. Cell-mediated cytotoxicity against virus-infected target cells in humans. II. Interferon induction and activation of natural killer cells. *J. Immunol. Baltim. Md 1950* **121,** 532–538 (1978).
191. Iwasaki, T. & Nozima, T. Defense mechanisms against primary influenza virus infection in mice. I. The roles of interferon and neutralizing antibodies and thymus dependence of interferon and antibody production. *J. Immunol. Baltim. Md 1950* **118,** 256–263 (1977).
192. García-Sastre, A. *et al.* The role of interferon in influenza virus tissue tropism. *J. Virol.* **72,** 8550–8558 (1998).
193. Price, G. E., Gaszewska-Mastarlarz, A. & Moskophidis, D. The role of alpha/beta and gamma interferons in development of immunity to influenza A virus in mice. *J. Virol.* **74,** 3996–4003 (2000).
194. Miao, H. *et al.* Quantifying the early immune response and adaptive immune response kinetics in mice infected with influenza A virus. *J. Virol.* **84,** 6687–6698 (2010).
195. Johansson, B. E., Bucher, D. J. & Kilbourne, E. D. Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. *J. Virol.* **63,** 1239–1246 (1989).
196. Tripp, R. A., Sarawar, S. R. & Doherty, P. C. Characteristics of the influenza virus-specific CD8+ T cell response in mice homozygous for disruption of the H-2IAb gene. *J. Immunol. Baltim. Md 1950* **155,** 2955–2959 (1995).
197. Topham, D. J., Tripp, R. A., Sarawar, S. R., Sangster, M. Y. & Doherty, P. C. Immune CD4+ T cells promote the clearance of influenza virus from major histocompatibility complex class II -/- respiratory epithelium. *J. Virol.* **70,** 1288–1291 (1996).

198. Topham, D. J., Tripp, R. A., Sarawar, S. R., Sangster, M. Y. & Doherty, P. C. Immune CD4+ T cells promote the clearance of influenza virus from major histocompatibility complex class II -/- respiratory epithelium. *J. Virol.* **70,** 1288–1291 (1996).
199. Van Riel, D. *et al.* Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. *Am. J. Pathol.* **171,** 1215–1223 (2007).
200. Mozdzanowska, K., Furchner, M., Maiese, K. & Gerhard, W. CD4+ T cells are ineffective in clearing a pulmonary infection with influenza type A virus in the absence of B cells. *Virology* **239,** 217–225 (1997).
201. Graham, M. B. & Braciale, T. J. Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J. Exp. Med.* **186,** 2063–2068 (1997).
202. Hou, S., Doherty, P. C., Zijlstra, M., Jaenisch, R. & Katz, J. M. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. *J. Immunol. Baltim. Md 1950* **149,** 1319–1325 (1992).
203. Handel, A., Longini, I. M., Jr & Antia, R. Neuraminidase inhibitor resistance in influenza: assessing the danger of its generation and spread. *PLoS Comput. Biol.* **3,** e240 (2007).
204. Baccam, P., Beauchemin, C., Macken, C. A., Hayden, F. G. & Perelson, A. S. Kinetics of influenza A virus infection in humans. *J. Virol.* **80,** 7590–7599 (2006).
205. Francis, T. & Stuart-Harris, C. H. STUDIES ON THE NASAL HISTOLOGY OF EPIDEMIC INFLUENZA VIRUS INFECTION IN THE FERRET : I. THE DEVELOPMENT AND REPAIR OF THE NASAL LESION. *J. Exp. Med.* **68,** 789–802 (1938).
206. Boivin, G., Goyette, N. & Bernatchez, H. Prolonged excretion of amantadine-resistant influenza a virus quasi species after cessation of antiviral therapy in an immunocompromised patient. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **34,** E23–25 (2002).
207. Rocha, E. *et al.* Antigenic and genetic variation in influenza A (H1N1) virus isolates recovered from a persistently infected immunodeficient child. *J. Virol.* **65,** 2340–2350 (1991).
208. Van den Brand, J. M. A. *et al.* Comparison of Temporal and Spatial Dynamics of Seasonal H3N2, Pandemic H1N1 and Highly Pathogenic Avian Influenza H5N1 Virus Infections in Ferrets. *PLoS One* **7,** (2012).
209. Kris, R. M., Yetter, R. A., Cogliano, R., Ramphal, R. & Small, P. A. Passive serum antibody causes temporary recovery from influenza virus infection of the nose, trachea and lung of nude mice. *Immunology* **63,** 349–353 (1988).
210. Iwasaki, T. & Nozima, T. Defense mechanisms against primary influenza virus infection in mice. I. The roles of interferon and neutralizing antibodies and thymus dependence of interferon and antibody production. *J. Immunol. Baltim. Md 1950* **118,** 256–263 (1977).

211. Handel, A., Longini, I. M. & Antia, R. Towards a quantitative understanding of the within-host dynamics of influenza A infections. *J. R. Soc. Interface* **7,** 35–47 (2010).
212. Miao, H. *et al.* Quantifying the Early Immune Response and Adaptive Immune Response Kinetics in Mice Infected with Influenza A Virus. *J. Virol.* **84,** 6687–6698 (2010).
213. Lee, H. Y. *et al.* Simulation and prediction of the adaptive immune response to influenza A virus infection. *J. Virol.* **83,** 7151–7165 (2009).
214. Lalvani, A. *et al.* Rapid effector function in CD8+ memory T cells. *J. Exp. Med.* **186,** 859–865 (1997).
215. Topham, D. J., Castrucci, M. R., Wingo, F. S., Belz, G. T. & Doherty, P. C. The Role of Antigen in the Localization of Naive, Acutely Activated, and Memory CD8+ T Cells to the Lung During Influenza Pneumonia. *J. Immunol.* **167,** 6983 –6990 (2001).
216. Doherty, P. C., Effros, R. B. & Bennink, J. Heterogeneity of the cytotoxic response of thymus-derived lymphocytes after immunization with influenza viruses. *Proc. Natl. Acad. Sci. U. S. A.* **74,** 1209–1213 (1977).
217. Effros, R. B., Doherty, P. C., Gerhard, W. & Bennink, J. Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses. *J. Exp. Med.* **145,** 557–568 (1977).
218. SCHULMAN, J. L. & KILBOURNE, E. D. INDUCTION OF PARTIAL SPECIFIC HETEROTYPIC IMMUNITY IN MICE BY A SINGLE INFECTION WITH INFLUENZA A VIRUS. *J. Bacteriol.* **89,** 170–174 (1965).
219. Taylor, P. M. & Askonas, B. A. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective in vivo. *Immunology* **58,** 417–420 (1986).
220. Lukacher, A. E., Braciale, V. L. & Braciale, T. J. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.* **160,** 814–826 (1984).
221. Yap, K. L. & Ada, G. L. The recovery of mice from influenza A virus infection: adoptive transfer of immunity with influenza virus-specific cytotoxic T lymphocytes recognizing a common virion antigen. *Scand. J. Immunol.* **8,** 413–420 (1978).
222. Liang, S., Mozdzanowska, K., Palladino, G. & Gerhard, W. Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. *J. Immunol. Baltim. Md 1950* **152,** 1653–1661 (1994).
223. McMichael, A. J., Gotch, F. M., Noble, G. R. & Beare, P. A. Cytotoxic T-cell immunity to influenza. *N. Engl. J. Med.* **309,** 13–17 (1983).
224. Sonoguchi, T., Naito, H., Hara, M., Takeuchi, Y. & Fukumi, H. Cross-subtype protection in humans during sequential, overlapping, and/or concurrent epidemics caused by H3N2 and H1N1 influenza viruses. *J. Infect. Dis.* **151,** 81–88 (1985).

225. Frank, A. L., Taber, L. H. & Wells, J. M. Individuals infected with two subtypes of influenza A virus in the same season. *J. Infect. Dis.* **147,** 120–124 (1983).
226. Gotch, F., McMichael, A., Smith, G. & Moss, B. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. *J. Exp. Med.* **165,** 408–416 (1987).
227. Squires, R. B. *et al.* Influenza research database: an integrated bioinformatics resource for influenza research and surveillance. *Influenza Other Respir. Viruses* **6,** 404–416 (2012).
228. Voeten, J. T. *et al.* Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *J. Virol.* **74,** 6800–6807 (2000).
229. Boon, A. C. M. *et al.* Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *J. Virol.* **76,** 2567–2572 (2002).
230. Rimmelzwaan, G. F. *et al.* Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *Virus Res.* **103,** 97–100 (2004).
231. Tu, W. *et al.* Cytotoxic T lymphocytes established by seasonal human influenza cross-react against 2009 pandemic H1N1 influenza virus. *J. Virol.* **84,** 6527–6535 (2010).
232. Lee, L. Y.-H. *et al.* Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *J. Clin. Invest.* **118,** 3478–3490 (2008).
233. McMichael, A. J., Gotch, F. M., Dongworth, D. W., Clark, A. & Potter, C. W. Declining T-cell immunity to influenza, 1977-82. *Lancet* **2,** 762–764 (1983).
234. Liang, S., Mozdzanowska, K., Palladino, G. & Gerhard, W. Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. *J. Immunol. Baltim. Md 1950* **152,** 1653–1661 (1994).
235. Hogan, R. J. *et al.* Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J. Immunol. Baltim. Md 1950* **166,** 1813–1822 (2001).
236. Kamperschroer, C., Dibble, J. P., Meents, D. L., Schwartzberg, P. L. & Swain, S. L. SAP is required for Th cell function and for immunity to influenza. *J. Immunol. Baltim. Md 1950* **177,** 5317–5327 (2006).
237. Seder, R. A. & Ahmed, R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat. Immunol.* **4,** 835–842 (2003).
238. McKinstry, K. K. *et al.* Memory CD4+ T cells protect against influenza through multiple synergizing mechanisms. *J. Clin. Invest.* **122,** 2847–2856 (2012).
239. Thomas, P. G. *et al.* Physiological numbers of CD4+ T cells generate weak recall responses following influenza virus challenge. *J. Immunol. Baltim. Md 1950* **184,** 1721–1727 (2010).

240. Brown, D. M., Dilzer, A. M., Meents, D. L. & Swain, S. L. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. *J. Immunol. Baltim. Md 1950* **177,** 2888–2898 (2006).
241. Mendelman, P. M. *et al.* Live attenuated influenza vaccine induces cross-reactive antibody responses in children against an a/Fujian/411/2002-like H3N2 antigenic variant strain. *Pediatr. Infect. Dis. J.* **23,** 1053–1055 (2004).
242. Mbawuike, I. N., Six, H. R., Cate, T. R. & Couch, R. B. Vaccination with inactivated influenza A virus during pregnancy protects neonatal mice against lethal challenge by influenza A viruses representing three subtypes. *J. Virol.* **64,** 1370–1374 (1990).
243. Okuno, Y., Isegawa, Y., Sasao, F. & Ueda, S. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J. Virol.* **67,** 2552–2558 (1993).
244. Ekiert, D. C. *et al.* Antibody recognition of a highly conserved influenza virus epitope: implications for universal prevention and therapy. *Science* **324,** 246–251 (2009).
245. Wrammert, J. *et al.* Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J. Exp. Med.* **208,** 181–193 (2011).
246. Corti, D. *et al.* Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. *J. Clin. Invest.* **120,** 1663–1673 (2010).
247. Corti, D. *et al.* A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* **333,** 850–856 (2011).
248. Wei, C.-J. *et al.* Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. *Science* **329,** 1060–1064 (2010).
249. Sui, J. *et al.* Structural and Functional Bases for Broad-Spectrum Neutralization of Avian and Human Influenza A Viruses. *Nat. Struct. X0026 Mol. Biol.* **16,** 265–273 (2009).
250. Steel, J. *et al.* Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio* **1,** (2010).
251. Black, R. A., Rota, P. A., Gorodkova, N., Klenk, H. D. & Kendal, A. P. Antibody response to the M2 protein of influenza A virus expressed in insect cells. *J. Gen. Virol.* **74 (Pt 1),** 143–146 (1993).
252. Feng, J. *et al.* Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. *Virol. J.* **3,** 102 (2006).
253. Treanor, J. J., Tierney, E. L., Zebedee, S. L., Lamb, R. A. & Murphy, B. R. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. *J. Virol.* **64,** 1375–1377 (1990).
254. Tissot, A. C. *et al.* Versatile virus-like particle carrier for epitope based vaccines. *PloS One* **5,** e9809 (2010).

255. Zhao, G. *et al.* An M2e-based multiple antigenic peptide vaccine protects mice from lethal challenge with divergent H5N1 influenza viruses. *Virol. J.* **7,** 9 (2010).
256. Slepushkin, V. A. *et al.* Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. *Vaccine* **13,** 1399–1402 (1995).
257. Frace, A. M., Klimov, A. I., Rowe, T., Black, R. A. & Katz, J. M. Modified M2 proteins produce heterotypic immunity against influenza A virus. *Vaccine* **17,** 2237–2244 (1999).
258. Mozdzanowska, K. *et al.* Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. *Vaccine* **21,** 2616–2626 (2003).
259. Zebedee, S. L. & Lamb, R. A. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J. Virol.* **62,** 2762–2772 (1988).
260. Jegerlehner, A., Schmitz, N., Storni, T. & Bachmann, M. F. Influenza A Vaccine Based on the Extracellular Domain of M2: Weak Protection Mediated via Antibody-Dependent NK Cell Activity. *J. Immunol.* **172,** 5598–5605 (2004).
261. Tompkins, S. M. *et al.* Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. *Emerg. Infect. Dis.* **13,** 426–435 (2007).
262. El Bakkouri, K. *et al.* Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. *J. Immunol. Baltim. Md 1950* **186,** 1022–1031 (2011).
263. Shu, L. L., Bean, W. J. & Webster, R. G. Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. *J. Virol.* **67,** 2723–2729 (1993).
264. Lamere, M. W. *et al.* Regulation of antinucleoprotein IgG by systemic vaccination and its effect on influenza virus clearance. *J. Virol.* **85,** 5027–5035 (2011).
265. Nguyen, H. H. *et al.* Heterosubtypic immunity to influenza A virus infection requires a properly diversified antibody repertoire. *J. Virol.* **81,** 9331–9338 (2007).
266. Carragher, D. M., Kaminski, D. A., Moquin, A., Hartson, L. & Randall, T. D. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *J. Immunol. Baltim. Md 1950* **181,** 4168–4176 (2008).
267. Wraith, D. C., Vessey, A. E. & Askonas, B. A. Purified influenza virus nucleoprotein protects mice from lethal infection. *J. Gen. Virol.* **68 (Pt 2),** 433–440 (1987).
268. LaMere, M. W. *et al.* Contributions of Antinucleoprotein IgG to Heterosubtypic Immunity against Influenza Virus. *J. Immunol. Baltim. Md 1950* **186,** 4331–4339 (2011).
269. Aung, S., Rutigliano, J. A. & Graham, B. S. Alternative Mechanisms of Respiratory Syncytial Virus Clearance in Perforin Knockout Mice Lead to Enhanced Disease. *J. Virol.* **75,** 9918–9924 (2001).
270. Wiley, J. A., Cerwenka, A., Harkema, J. R., Dutton, R. W. & Harmsen, A. G. Production of interferon-gamma by influenza hemagglutinin-specific CD8 effector

T cells influences the development of pulmonary immunopathology. *Am. J. Pathol.* **158,** 119–130 (2001).

271. Influenza virus-specific CD4+ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. *J. Exp. Med.* **180,** 1273–1282 (1994).
272. Graham, M. B., Braciale, V. L. & Braciale, T. J. Influenza virus-specific CD4+ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. *J. Exp. Med.* **180,** 1273–1282 (1994).
273. Bermejo-Martin, J. F. *et al.* Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza. *Crit. Care Lond. Engl.* **13,** R201 (2009).
274. Crowe, C. R. *et al.* Critical Role of IL-17RA in Immunopathology of Influenza Infection. *J. Immunol.* **183,** 5301 –5310 (2009).
275. Reinhardt, R. L., Khoruts, A., Merica, R., Zell, T. & Jenkins, M. K. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* **410,** 101–105 (2001).
276. Sallusto, F., Lenig, D., Förster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401,** 708–712 (1999).
277. Harris, N. L., Watt, V., Ronchese, F. & Le Gros, G. Differential T cell function and fate in lymph node and nonlymphoid tissues. *J. Exp. Med.* **195,** 317–326 (2002).
278. Masopust, D., Vezys, V., Marzo, A. L. & Lefrançois, L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* **291,** 2413–2417 (2001).
279. Bennink, J., Effros, R. B. & Doherty, P. C. Influenzal pneumonia: early appearance of cross-reactive T cells in lungs of mice primed with heterologous type A viruses. *Immunology* **35,** 503–509 (1978).
280. Flynn, K. J. *et al.* Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity* **8,** 683–691 (1998).
281. Chvatchko, Y., Kosco-Vilbois, M. H., Herren, S., Lefort, J. & Bonnefoy, J. Y. Germinal center formation and local immunoglobulin E (IgE) production in the lung after an airway antigenic challenge. *J. Exp. Med.* **184,** 2353–2360 (1996).
282. Borron, P. J. *et al.* Recombinant rat surfactant-associated protein D inhibits human T lymphocyte proliferation and IL-2 production. *J. Immunol. Baltim. Md 1950* **161,** 4599–4603 (1998).
283. Wiley, J. A., Hogan, R. J., Woodland, D. L. & Harmsen, A. G. Antigen-specific CD8(+) T cells persist in the upper respiratory tract following influenza virus infection. *J. Immunol. Baltim. Md 1950* **167,** 3293–3299 (2001).
284. Hogan, R. J. *et al.* Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J. Immunol. Baltim. Md 1950* **166,** 1813–1822 (2001).
285. Woodland, D. L., Hogan, R. J. & Zhong, W. Cellular immunity and memory to respiratory virus infections. *Immunol. Res.* **24,** 53–67 (2001).
286. Wherry, E. J. *et al.* Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* **4,** 225–234 (2003).

287. Topham, D. J., Castrucci, M. R., Wingo, F. S., Belz, G. T. & Doherty, P. C. The role of antigen in the localization of naive, acutely activated, and memory CD8(+) T cells to the lung during influenza pneumonia. *J. Immunol. Baltim. Md 1950* **167,** 6983–6990 (2001).
288. Hogan, R. J. *et al.* Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J. Immunol. Baltim. Md 1950* **166,** 1813–1822 (2001).
289. Ely, K. H., Roberts, A. D. & Woodland, D. L. Cutting edge: effector memory CD8+ T cells in the lung airways retain the potential to mediate recall responses. *J. Immunol. Baltim. Md 1950* **171,** 3338–3342 (2003).
290. Hogan, R. J. *et al.* Long-term maintenance of virus-specific effector memory CD8+ T cells in the lung airways depends on proliferation. *J. Immunol. Baltim. Md 1950* **169,** 4976–4981 (2002).
291. Mrusek, S., Vallbracht, S. & Ehl, S. The impact of splenectomy on antiviral T cell memory in mice. *Int. Immunol.* **17,** 27–33 (2005).
292. Lund, F. E. *et al.* Lymphotoxin-alpha-deficient mice make delayed, but effective, T and B cell responses to influenza. *J. Immunol. Baltim. Md 1950* **169,** 5236–5243 (2002).
293. Wiley, J. A., Tighe, M. P. & Harmsen, A. G. Upper respiratory tract resistance to influenza infection is not prevented by the absence of either nasal-associated lymphoid tissue or cervical lymph nodes. *J. Immunol. Baltim. Md 1950* **175,** 3186–3196 (2005).
294. Nguyen, H. H. *et al.* Heterosubtypic Immunity to Lethal Influenza A Virus Infection Is Associated with Virus-Specific CD8+Cytotoxic T Lymphocyte Responses Induced in Mucosa-Associated Tissues. *Virology* **254,** 50–60 (1999).
295. Woodland, D. L., Hogan, R. J. & Zhong, W. Cellular immunity and memory to respiratory virus infections. *Immunol. Res.* **24,** 53–67 (2001).
296. Ely, K. H. *et al.* Nonspecific recruitment of memory CD8+ T cells to the lung airways during respiratory virus infections. *J. Immunol. Baltim. Md 1950* **170,** 1423–1429 (2003).
297. Becker, T. C. *et al.* Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J. Exp. Med.* **195,** 1541–1548 (2002).
298. Tan, J. T. *et al.* Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J. Exp. Med.* **195,** 1523–1532 (2002).
299. Ku, C. C., Murakami, M., Sakamoto, A., Kappler, J. & Marrack, P. Control of homeostasis of CD8+ memory T cells by opposing cytokines. *Science* **288,** 675–678 (2000).
300. Ely, K. H., Cookenham, T., Roberts, A. D. & Woodland, D. L. Memory T cell populations in the lung airways are maintained by continual recruitment. *J. Immunol. Baltim. Md 1950* **176,** 537–543 (2006).

301. Beyer, M. *et al.* Sustained increases in numbers of pulmonary dendritic cells after respiratory syncytial virus infection. *J. Allergy Clin. Immunol.* **113,** 127–133 (2004).
302. Dahl, M. E., Dabbagh, K., Liggitt, D., Kim, S. & Lewis, D. B. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat. Immunol.* **5,** 337–343 (2004).
303. Kirby, A. C., Newton, D. J., Carding, S. R. & Kaye, P. M. Pulmonary dendritic cells and alveolar macrophages are regulated by gammadelta T cells during the resolution of S. pneumoniae-induced inflammation. *J. Pathol.* **212,** 29–37 (2007).
304. Thepen, T., Kraal, G. & Holt, P. G. The role of alveolar macrophages in regulation of lung inflammation. *Ann. N. Y. Acad. Sci.* **725,** 200–206 (1994).
305. Bilyk, N. & Holt, P. G. Inhibition of the immunosuppressive activity of resident pulmonary alveolar macrophages by granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **177,** 1773–1777 (1993).
306. Kirby, A. C., Raynes, J. G. & Kaye, P. M. CD11b regulates recruitment of alveolar macrophages but not pulmonary dendritic cells after pneumococcal challenge. *J. Infect. Dis.* **193,** 205–213 (2006).
307. Guillot, L. *et al.* Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J. Biol. Chem.* **280,** 5571–5580 (2005).
308. Goulding, J. *et al.* Respiratory infections: do we ever recover? *Proc. Am. Thorac. Soc.* **4,** 618–625 (2007).
309. McMichael, A. J., Gotch, F. M., Dongworth, D. W., Clark, A. & Potter, C. W. Declining T-cell immunity to influenza, 1977-82. *Lancet* **2,** 762–764 (1983).
310. Roberts, A. D., Ely, K. H. & Woodland, D. L. Differential contributions of central and effector memory T cells to recall responses. *J. Exp. Med.* **202,** 123–133 (2005).
311. Low, W. *et al.* Internal Ribosome Entry Site Regulates Translation of Kaposi's Sarcoma-Associated Herpesvirus FLICE Inhibitory Protein. *J. Virol.* **75,** 2938–2945 (2001).
312. Efklidou, S., Bailey, R., Field, N., Noursadeghi, M. & Collins, M. K. vFLIP from KSHV inhibits anoikis of primary endothelial cells. *J. Cell Sci.* **121,** 450–457 (2008).
313. Karwacz, K. *et al.* Nonintegrating Lentivector Vaccines Stimulate Prolonged T-Cell and Antibody Responses and Are Effective in Tumor Therapy. *J. Virol.* **83,** 3094–3103 (2009).
314. Rowe, H. M. *et al.* Expression of vFLIP in a lentiviral vaccine vector activates NF-{kappa}B, matures dendritic cells, and increases CD8+ T-cell responses. *J. Virol.* **83,** 1555–1562 (2009).
315. Arce, F. *et al.* Selective ERK activation differentiates mouse and human tolerogenic dendritic cells, expands antigen-specific regulatory T cells, and suppresses experimental inflammatory arthritis. *Arthritis Rheum.* **63,** 84–95 (2011).

316. Papagatsias, T. *et al.* Activity of different vaccine-associated promoter elements in human dendritic cells. *Immunol. Lett.* **115,** 117–125 (2008).
317. Papagatsias, T. Activity of different vaccine-associated promoter elements in human dendritic cells. *Immunol. Lett.* **115,** 117–125 (2008).
318. Brown, B. D. *et al.* A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood* **110,** 4144–4152 (2007).
319. Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59–74 (1977).
320. Shimizu, A. *et al.* Kaposi's sarcoma-associated herpesvirus vFLIP and human T cell lymphotropic virus type 1 Tax oncogenic proteins activate IkappaB kinase subunit gamma by different mechanisms independent of the physiological cytokine-induced pathways. *J. Virol.* **85,** 7444–7448 (2011).
321. Talmor, M. *et al.* Generation or large numbers of immature and mature dendritic cells from rat bone marrow cultures. *Eur. J. Immunol.* **28,** 811–817 (1998).
322. Buckley, S. M. K. *et al.* Lentiviral transduction of the murine lung provides efficient pseudotype and developmental stage-dependent cell-specific transgene expression. *Gene Ther.* **15,** 1167–1175 (2008).
323. Van Rijt, L. S. *et al.* In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J. Exp. Med.* **201,** 981–991 (2005).
324. Steinman, R. M. & Witmer, M. D. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. U. S. A.* **75,** 5132–5136 (1978).
325. Hawiger, D. *et al.* Dendritic Cells Induce Peripheral T Cell Unresponsiveness under Steady State Conditions in Vivo. *J. Exp. Med.* **194,** 769–780 (2001).
326. Probst, H. C., Lagnel, J., Kollias, G. & van den Broek, M. Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8+ T cell tolerance. *Immunity* **18,** 713–720 (2003).
327. Bonifaz, L. *et al.* Efficient Targeting of Protein Antigen to the Dendritic Cell Receptor DEC-205 in the Steady State Leads to Antigen Presentation on Major Histocompatibility Complex Class I Products and Peripheral CD8+ T Cell Tolerance. *J. Exp. Med.* **196,** 1627–1638 (2002).
328. Tarbell, K. V. *et al.* Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. *J. Exp. Med.* **204,** 191–201 (2007).
329. Koya, R. C. *et al.* Making dendritic cells from the inside out: lentiviral vector-mediated gene delivery of granulocyte-macrophage colony-stimulating factor and interleukin 4 into CD14+ monocytes generates dendritic cells in vitro. *Hum. Gene Ther.* **15,** 733–748 (2004).

330. Koya, R. C. *et al.* Potent maturation of monocyte-derived dendritic cells after CD40L lentiviral gene delivery. *J. Immunother. Hagerstown Md 1997* **26,** 451–460 (2003).
331. Kobayashi, M., Takaori-Kondo, A., Fukunaga, K., Miyoshi, H. & Uchiyama, T. Lentiviral gp34/OX40L gene transfer into dendritic cells facilitates alloreactive CD4+ T-cell response in vitro. *Int. J. Hematol.* **79,** 377–383 (2004).
332. Kim, J. H. *et al.* Induction of therapeutic antitumor immunity by in vivo administration of a lentiviral vaccine. *Hum. Gene Ther.* **16,** 1255–1266 (2005).
333. Yamamoto, M. *et al.* Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* **301,** 640–643 (2003).
334. Banerjee, A., Gugasyan, R., McMahon, M. & Gerondakis, S. Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 3274–3279 (2006).
335. Arce, F. *et al.* Selective ERK activation differentiates mouse and human tolerogenic dendritic cells, expands antigen-specific regulatory T cells, and suppresses experimental inflammatory arthritis. *Arthritis Rheum.* **63,** 84–95 (2011).
336. Saraiva, M. & O'Garra, A. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* **10,** 170–181 (2010).
337. Arrighi, J. F., Rebsamen, M., Rousset, F., Kindler, V. & Hauser, C. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. *J. Immunol. Baltim. Md 1950* **166,** 3837–3845 (2001).
338. Nakahara, T. *et al.* Role of c-Jun N-terminal kinase on lipopolysaccharide induced maturation of human monocyte-derived dendritic cells. *Int. Immunol.* **16,** 1701–1709 (2004).
339. Xiao, W. Advances in NF-kappaB signaling transduction and transcription. *Cell. Mol. Immunol.* **1,** 425–435 (2004).
340. Kawai, T. & Akira, S. Toll-like receptor and RIG-I-like receptor signaling. *Ann. N. Y. Acad. Sci.* **1143,** 1–20 (2008).
341. Senftleben, U. *et al.* Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* **293,** 1495–1499 (2001).
342. Escors, D. *et al.* Targeting dendritic cell signaling to regulate the response to immunization. *Blood* **111,** 3050–3061 (2008).
343. Andreakos, E., Williams, R. O., Wales, J., Foxwell, B. M. & Feldmann, M. Activation of NF-kappaB by the intracellular expression of NF-kappaB-inducing kinase acts as a powerful vaccine adjuvant. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 14459–14464 (2006).
344. Jing, Z. *et al.* Chromosome 1 Open Reading Frame 190 Promotes Activation of NF-κB Canonical Pathway and Resistance of Dendritic Cells to Tumor-Associated Inhibition In Vitro. *J. Immunol.* **185,** 6719–6727 (2010).

345. Heyninck, K. & Beyaert, R. The cytokine-inducible zinc finger protein A20 inhibits IL-1-induced NF-kappaB activation at the level of TRAF6. *FEBS Lett.* **442,** 147–150 (1999).
346. Song, X.-T. *et al.* A20 is an antigen presentation attenuator, and its inhibition overcomes regulatory T cell-mediated suppression. *Nat. Med.* **14,** 258–265 (2008).
347. Breckpot, K. *et al.* Attenuated expression of A20 markedly increases the efficacy of double-stranded RNA-activated dendritic cells as an anti-cancer vaccine. *J. Immunol. Baltim. Md 1950* **182,** 860–870 (2009).
348. Sun, Q., Zachariah, S. & Chaudhary, P. M. The human herpes virus 8-encoded viral FLICE-inhibitory protein induces cellular transformation via NF-kappaB activation. *J. Biol. Chem.* **278,** 52437–52445 (2003).
349. Matta, H., Sun, Q., Moses, G. & Chaudhary, P. M. Molecular genetic analysis of human herpes virus 8-encoded viral FLICE inhibitory protein-induced NF-kappaB activation. *J. Biol. Chem.* **278,** 52406–52411 (2003).
350. Field, N. *et al.* KSHV vFLIP binds to IKK-gamma to activate IKK. *J. Cell Sci.* **116,** 3721–3728 (2003).
351. Liu, L. *et al.* The human herpes virus 8-encoded viral FLICE inhibitory protein physically associates with and persistently activates the Ikappa B kinase complex. *J. Biol. Chem.* **277,** 13745–13751 (2002).
352. Bagnéris, C. *et al.* Crystal structure of a vFlip-IKKgamma complex: insights into viral activation of the IKK signalosome. *Mol. Cell* **30,** 620–631 (2008).
353. Matta, H. & Chaudhary, P. M. Activation of alternative NF-kappa B pathway by human herpes virus 8-encoded Fas-associated death domain-like IL-1 beta-converting enzyme inhibitory protein (vFLIP). *Proc. Natl. Acad. Sci. U. S. A.* **101,** 9399–9404 (2004).
354. Punj, V., Matta, H. & Chaudhary, P. M. A computational profiling of changes in gene expression and transcription factors induced by vFLIP K13 in primary effusion lymphoma. *PloS One* **7,** e37498 (2012).
355. Punj, V., Matta, H., Schamus, S. & Chaudhary, P. M. Integrated microarray and multiplex cytokine analyses of Kaposi's Sarcoma Associated Herpesvirus viral FLICE Inhibitory Protein K13 affected genes and cytokines in human blood vascular endothelial cells. *BMC Med. Genomics* **2,** 50 (2009).
356. Shimizu, A. *et al.* Kaposi's sarcoma-associated herpesvirus vFLIP and human T cell lymphotropic virus type 1 Tax oncogenic proteins activate IkappaB kinase subunit gamma by different mechanisms independent of the physiological cytokine-induced pathways. *J. Virol.* **85,** 7444–7448 (2011).
357. Matta, H. *et al.* Kaposi's sarcoma associated herpesvirus encoded viral FLICE inhibitory protein K13 activates NF-κB pathway independent of TRAF6, TAK1 and LUBAC. *PloS One* **7,** e36601 (2012).
358. Matta, H. *et al.* A20 is induced by Kaposi sarcoma-associated herpesvirus-encoded viral FLICE inhibitory protein (vFLIP) K13 and blocks K13-induced nuclear

factor-kappaB in a negative feedback manner. *J. Biol. Chem.* **286,** 21555–21564 (2011).

359. Rowe, H. M. *et al.* Expression of vFLIP in a lentiviral vaccine vector activates NF-{kappa}B, matures dendritic cells, and increases CD8+ T-cell responses. *J. Virol.* **83,** 1555–1562 (2009).
360. Dai, B. *et al.* HIV-1 Gag-specific immunity induced by a lentivector-based vaccine directed to dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 20382–20387 (2009).
361. Buffa, V. *et al.* Evaluation of a self-inactivating lentiviral vector expressing simian immunodeficiency virus gag for induction of specific immune responses in vitro and in vivo. *Viral Immunol.* **19,** 690–701 (2006).
362. Beignon, A.-S. *et al.* Lentiviral vector-based prime/boost vaccination against AIDS: pilot study shows protection against Simian immunodeficiency virus SIVmac251 challenge in macaques. *J. Virol.* **83,** 10963–10974 (2009).
363. Fu, T. M., Friedman, A., Ulmer, J. B., Liu, M. A. & Donnelly, J. J. Protective cellular immunity: cytotoxic T-lymphocyte responses against dominant and recessive epitopes of influenza virus nucleoprotein induced by DNA immunization. *J. Virol.* **71,** 2715–2721 (1997).
364. Fu, T. M. *et al.* Dose dependence of CTL precursor frequency induced by a DNA vaccine and correlation with protective immunity against influenza virus challenge. *J. Immunol. Baltim. Md 1950* **162,** 4163–4170 (1999).
365. Ulmer, J. B. *et al.* Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. *J. Virol.* **72,** 5648–5653 (1998).
366. Robinson, H. L. *et al.* DNA immunization for influenza virus: studies using hemagglutinin- and nucleoprotein-expressing DNAs. *J. Infect. Dis.* **176 Suppl 1,** S50–55 (1997).
367. Chen, Z. *et al.* Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein. *J. Gen. Virol.* **80 (Pt 10),** 2559–2564 (1999).
368. Chen, Z. *et al.* Comparison of the ability of viral protein-expressing plasmid DNAs to protect against influenza. *Vaccine* **16,** 1544–1549 (1998).
369. Saha, S. *et al.* A fused gene of nucleoprotein (NP) and herpes simplex virus genes (VP22) induces highly protective immunity against different subtypes of influenza virus. *Virology* **354,** 48–57 (2006).
370. Roy, S. *et al.* Partial protection against H5N1 influenza in mice with a single dose of a chimpanzee adenovirus vector expressing nucleoprotein. *Vaccine* **25,** 6845–6851 (2007).
371. Tsuji, M. *et al.* Recombinant Sindbis viruses expressing a cytotoxic T-lymphocyte epitope of a malaria parasite or of influenza virus elicit protection against the corresponding pathogen in mice. *J. Virol.* **72,** 6907–6910 (1998).
372. Laddy, D. J. *et al.* Heterosubtypic protection against pathogenic human and avian influenza viruses via in vivo electroporation of synthetic consensus DNA antigens. *PloS One* **3,** e2517 (2008).

373. Bender, B. S. *et al.* Immunogenicity and efficacy of DNA vaccines encoding influenza A proteins in aged mice. *Vaccine* **16,** 1748–1755 (1998).
374. Ulmer, J. B. *et al.* Protective immunity by intramuscular injection of low doses of influenza virus DNA vaccines. *Vaccine* **12,** 1541–1544 (1994).
375. Epstein, S. L. *et al.* DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice. *Emerg. Infect. Dis.* **8,** 796–801 (2002).
376. Bot, A., Bot, S., García-Sastre, A. & Bona, C. Protective cellular immunity against influenza virus induced by plasmid inoculation of newborn mice. *Dev. Immunol.* **5,** 197–210 (1998).
377. Epstein, S. L. *et al.* Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. *Vaccine* **23,** 5404–5410 (2005).
378. Altstein, A. D. *et al.* Immunization with influenza A NP-expressing vaccinia virus recombinant protects mice against experimental infection with human and avian influenza viruses. *Arch. Virol.* **151,** 921–931 (2006).
379. Moraes, T. J., Lin, G. H. Y., Wen, T. & Watts, T. H. Incorporation of 4-1BB ligand into an adenovirus vaccine vector increases the number of functional antigen-specific CD8 T cells and enhances the duration of protection against influenza-induced respiratory disease. *Vaccine* **29,** 6301–6312 (2011).
380. MacLeod, M. K. L. *et al.* Vaccine adjuvants aluminum and monophosphoryl lipid A provide distinct signals to generate protective cytotoxic memory CD8 T cells. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 7914–7919 (2011).
381. Jimenez, G. S. *et al.* Vaxfectin-formulated influenza DNA vaccines encoding NP and M2 viral proteins protect mice against lethal viral challenge. *Hum. Vaccin.* **3,** 157–164 (2007).
382. James, C. M. *et al.* Differential activities of alpha/beta IFN subtypes against influenza virus in vivo and enhancement of specific immune responses in DNA vaccinated mice expressing haemagglutinin and nucleoprotein. *Vaccine* **25,** 1856–1867 (2007).
383. Dégano, P., Schneider, J., Hannan, C. M., Gilbert, S. C. & Hill, A. V. Gene gun intradermal DNA immunization followed by boosting with modified vaccinia virus Ankara: enhanced CD8+ T cell immunogenicity and protective efficacy in the influenza and malaria models. *Vaccine* **18,** 623–632 (1999).
384. Woodland, D. L. & Randall, T. D. Anatomical features of anti-viral immunity in the respiratory tract. *Semin. Immunol.* **16,** 163–170 (2004).
385. Nguyen, H. H. *et al.* Heterosubtypic immunity to lethal influenza A virus infection is associated with virus-specific CD8(+) cytotoxic T lymphocyte responses induced in mucosa-associated tissues. *Virology* **254,** 50–60 (1999).
386. Dullaers, M. *et al.* Induction of effective therapeutic antitumor immunity by direct in vivo administration of lentiviral vectors. *Gene Ther.* **13,** 630–640 (2006).
387. Esslinger, C. *et al.* In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses. *J. Clin. Invest.* **111,** 1673–1681 (2003).

388. Iglesias, M. C. *et al.* Lentiviral vectors encoding HIV-1 polyepitopes induce broad CTL responses in vivo. *Mol. Ther. J. Am. Soc. Gene Ther.* **15,** 1203–1210 (2007).
389. Dai, B. *et al.* HIV-1 Gag-specific immunity induced by a lentivector-based vaccine directed to dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 20382–20387 (2009).
390. Masopust, D. Developing an HIV cytotoxic T-lymphocyte vaccine: issues of CD8 T-cell quantity, quality and location. *J. Intern. Med.* **265,** 125–137 (2009).
391. Nguyen, H. H. *et al.* Heterosubtypic Immunity to Lethal Influenza A Virus Infection Is Associated with Virus-Specific CD8+Cytotoxic T Lymphocyte Responses Induced in Mucosa-Associated Tissues. *Virology* **254,** 50–60 (1999).
392. Veres, T. Z. *et al.* Spatiotemporal and functional behavior of airway dendritic cells visualized by two-photon microscopy. *Am. J. Pathol.* **179,** 603–609 (2011).
393. BALBo, P., Silvestri, M., Rossi, G. A., Crimi, E. & Burastero, S. E. Differential role of CD80 and CD86 on alveolar macrophages in the presentation of allergen to T lymphocytes in asthma. *Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol.* **31,** 625–636 (2001).
394. Chelen, C. J. *et al.* Human alveolar macrophages present antigen ineffectively due to defective expression of B7 costimulatory cell surface molecules. *J. Clin. Invest.* **95,** 1415–1421 (1995).
395. Ettensohn, D. B., Lalor, P. A. & Roberts, N. J., Jr. Human alveolar macrophage regulation of lymphocyte proliferation. *Am. Rev. Respir. Dis.* **133,** 1091–1096 (1986).
396. Thepen, T., Kraal, G. & Holt, P. G. The role of alveolar macrophages in regulation of lung inflammation. *Ann. N. Y. Acad. Sci.* **725,** 200–206 (1994).
397. Lipscomb, M. F., Pollard, A. M. & Yates, J. L. A role for TGF-beta in the suppression by murine bronchoalveolar cells of lung dendritic cell initiated immune responses. *Reg. Immunol.* **5,** 151–157 (1993).
398. Holt, P. G. *et al.* Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J. Exp. Med.* **177,** 397–407 (1993).
399. Van Rooijen, N. The liposome-mediated macrophage 'suicide' technique. *J. Immunol. Methods* **124,** 1–6 (1989).
400. Strickland, D. H., Thepen, T., Kees, U. R., Kraal, G. & Holt, P. G. Regulation of T-cell function in lung tissue by pulmonary alveolar macrophages. *Immunology* **80,** 266–272 (1993).
401. Henning, L. N. *et al.* Pulmonary surfactant protein A regulates TLR expression and activity in human macrophages. *J. Immunol. Baltim. Md 1950* **180,** 7847–7858 (2008).
402. Yamada, C. *et al.* Surfactant protein A directly interacts with TLR4 and MD-2 and regulates inflammatory cellular response. Importance of supratrimeric oligomerization. *J. Biol. Chem.* **281,** 21771–21780 (2006).

403. Janssen, W. J. *et al.* Surfactant proteins A and D suppress alveolar macrophage phagocytosis via interaction with SIRP alpha. *Am. J. Respir. Crit. Care Med.* **178,** 158–167 (2008).
404. Ueno, K. *et al.* MUC1 mucin is a negative regulator of toll-like receptor signaling. *Am. J. Respir. Cell Mol. Biol.* **38,** 263–268 (2008).
405. Wright, G. J. *et al.* Lymphoid/neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. *Immunity* **13,** 233–242 (2000).
406. Fernandez, S., Jose, P., Avdiushko, M. G., Kaplan, A. M. & Cohen, D. A. Inhibition of IL-10 receptor function in alveolar macrophages by Toll-like receptor agonists. *J. Immunol. Baltim. Md 1950* **172,** 2613–2620 (2004).
407. Morris, D. G. *et al.* Loss of integrin alpha(v)beta6-mediated TGF-beta activation causes Mmp12-dependent emphysema. *Nature* **422,** 169–173 (2003).
408. Hoft, D. F. *et al.* Live and Inactivated Influenza Vaccines Induce Similar Humoral Responses, but Only Live Vaccines Induce Diverse T-Cell Responses in Young Children. *J. Infect. Dis.* **204,** 845–853 (2011).
409. Subbramanian, R. A., Basha, S., Shata, M. T., Brady, R. C. & Bernstein, D. I. Pandemic and seasonal H1N1 influenza hemagglutinin-specific T cell responses elicited by seasonal influenza vaccination. *Vaccine* **28,** 8258–8267 (2010).
410. Zeman, A. M. *et al.* Humoral and cellular immune responses in children given annual immunization with trivalent inactivated influenza vaccine. *Pediatr. Infect. Dis. J.* **26,** 107–115 (2007).
411. He, X.-S. *et al.* Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. *J. Virol.* **80,** 11756–11766 (2006).
412. Lynch, J. P., 3rd, Fishbein, M. & Echavarria, M. Adenovirus. *Semin. Respir. Crit. Care Med.* **32,** 494–511 (2011).
413. Yang, Y., Li, Q., Ertl, H. C. & Wilson, J. M. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* **69,** 2004–2015 (1995).
414. Van Ginkel, F. W. *et al.* Adenoviral gene delivery elicits distinct pulmonary-associated T helper cell responses to the vector and to its transgene. *J. Immunol. Baltim. Md 1950* **159,** 685–693 (1997).
415. Wang, J. *et al.* Single Mucosal, but Not Parenteral, Immunization with Recombinant Adenoviral-Based Vaccine Provides Potent Protection from Pulmonary Tuberculosis. *J. Immunol.* **173,** 6357–6365 (2004).
416. Price, G. E. *et al.* Single-dose mucosal immunization with a candidate universal influenza vaccine provides rapid protection from virulent H5N1, H3N2 and H1N1 viruses. *PLoS ONE* **5,** e13162 (2010).
417. Gerhard, W., Mozdzanowska, K., Furchner, M., Washko, G. & Maiese, K. Role of the B-cell response in recovery of mice from primary influenza virus infection. *Immunol. Rev.* **159,** 95–103 (1997).

418. Hochstein-Mintzel, V., Stickl, H. & Huber, H. C. Oral immunization against smallpox. *Dev. Biol. Stand.* **33,** 260–266 (1976).
419. Gherardi, M. M. & Esteban, M. Recombinant poxviruses as mucosal vaccine vectors. *J. Gen. Virol.* **86,** 2925–2936 (2005).
420. Gherardi, M. M., Pérez-Jiménez, E., Nájera, J. L. & Esteban, M. Induction of HIV immunity in the genital tract after intranasal delivery of a MVA vector: enhanced immunogenicity after DNA prime-modified vaccinia virus Ankara boost immunization schedule. *J. Immunol. Baltim. Md 1950* **172,** 6209–6220 (2004).
421. Manrique, M. *et al.* Nasal DNA-MVA SIV vaccination provides more significant protection from progression to AIDS than a similar intramuscular vaccination. *Mucosal Immunol.* **2,** 536–550 (2009).
422. Huneycutt, B. S. *et al.* Distribution of vesicular stomatitis virus proteins in the brains of BALB/c mice following intranasal inoculation: an immunohistochemical analysis. *Brain Res.* **635,** 81–95 (1994).
423. Wilson, S. R. *et al.* Intranasal immunization with recombinant vesicular stomatitis virus expressing murine cytomegalovirus glycoprotein B induces humoral and cellular immunity. *Comp. Med.* **58,** 129–139 (2008).
424. Egan, M. A. *et al.* Immunogenicity of attenuated vesicular stomatitis virus vectors expressing HIV type 1 Env and SIV Gag proteins: comparison of intranasal and intramuscular vaccination routes. *AIDS Res. Hum. Retroviruses* **20,** 989–1004 (2004).
425. Roberts, A. *et al.* Vaccination with a recombinant vesicular stomatitis virus expressing an influenza virus hemagglutinin provides complete protection from influenza virus challenge. *J. Virol.* **72,** 4704–4711 (1998).
426. Barefoot, B. E., Sample, C. J. & Ramsburg, E. A. Recombinant Vesicular Stomatitis Virus Expressing Influenza Nucleoprotein Induces CD8 T-Cell Responses That Enhance Antibody-Mediated Protection after Lethal Challenge with Influenza Virus. *Clin. Vaccine Immunol. CVI* **16,** 488–498 (2009).
427. Csaba, N., Garcia-Fuentes, M. & Alonso, M. J. Nanoparticles for nasal vaccination. *Adv. Drug Deliv. Rev.* **61,** 140–157 (2009).
428. T Hart, B. A. *et al.* Liposome-mediated peptide loading of MHC-DR molecules in vivo. *FEBS Lett.* **409,** 91–95 (1997).
429. Baca-Estrada, M. E. *et al.* Intranasal immunization with liposome-formulated Yersinia pestis vaccine enhances mucosal immune responses. *Vaccine* **18,** 2203–2211 (2000).
430. Sloat, B. R. & Cui, Z. Strong mucosal and systemic immunities induced by nasal immunization with anthrax protective antigen protein incorporated in liposome-protamine-DNA particles. *Pharm. Res.* **23,** 262–269 (2006).
431. Rosada, R. S. *et al.* Protection against tuberculosis by a single intranasal administration of DNA-hsp65 vaccine complexed with cationic liposomes. *BMC Immunol.* **9,** 38 (2008).

432. Hirosue, S., Kourtis, I. C., van der Vlies, A. J., Hubbell, J. A. & Swartz, M. A. Antigen delivery to dendritic cells by poly(propylene sulfide) nanoparticles with disulfide conjugated peptides: Cross-presentation and T cell activation. *Vaccine* **28,** 7897–7906 (2010).
433. Enose, Y. *et al.* Protection by intranasal immunization of a nef-deleted, nonpathogenic SHIV against intravaginal challenge with a heterologous pathogenic SHIV. *Virology* **298,** 306–316 (2002).
434. Goldman, M. J., Lee, P. S., Yang, J. S. & Wilson, J. M. Lentiviral vectors for gene therapy of cystic fibrosis. *Hum. Gene Ther.* **8,** 2261–2268 (1997).
435. Kobinger, G. P., Weiner, D. J., Yu, Q. C. & Wilson, J. M. Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nat. Biotechnol.* **19,** 225–230 (2001).
436. Limberis, M., Anson, D. S., Fuller, M. & Parsons, D. W. Recovery of airway cystic fibrosis transmembrane conductance regulator function in mice with cystic fibrosis after single-dose lentivirus-mediated gene transfer. *Hum. Gene Ther.* **13,** 1961–1970 (2002).
437. Stocker, A. G. *et al.* Single-dose lentiviral gene transfer for lifetime airway gene expression. *J. Gene Med.* **11,** 861–867 (2009).
438. Copreni, E., Penzo, M., Carrabino, S. & Conese, M. Lentivirus-mediated gene transfer to the respiratory epithelium: a promising approach to gene therapy of cystic fibrosis. *Gene Ther.* **11,** S67–S75 (print).
439. Malur, A. *et al.* Restoration of PPARγ reverses lipid accumulation in alveolar macrophages of GM-CSF knockout mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **300,** L73–80 (2011).
440. Selective induction of monocyte and not neutrophil-attracting chemokines after influenza A virus infection. *J. Exp. Med.* **184,** 1191–1196 (1996).
441. Kaufmann, A. *et al.* Defense against influenza A virus infection: essential role of the chemokine system. *Immunobiology* **204,** 603–613 (2001).
442. Buckley, S. M. K. *et al.* Lentiviral transduction of the murine lung provides efficient pseudotype and developmental stage-dependent cell-specific transgene expression. *Gene Ther.* **15,** 1167–1175 (2008).
443. Thepen, T., Van Rooijen, N. & Kraal, G. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. *J. Exp. Med.* **170,** 499–509 (1989).
444. Van Rijt, L. S. *et al.* In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J. Exp. Med.* **201,** 981–991 (2005).
445. Crowell, R. E., Heaphy, E., Valdez, Y. E., Mold, C. & Lehnert, B. E. Alveolar and interstitial macrophage populations in the murine lung. *Exp. Lung Res.* **18,** 435–446 (1992).
446. Kono, H. & Rock, K. L. How dying cells alert the immune system to danger. *Nat. Rev. Immunol.* **8,** 279–289 (2008).

447. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8,** 958–969 (2008).
448. Guth, A. M. *et al.* Lung environment determines unique phenotype of alveolar macrophages. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **296,** L936–946 (2009).
449. Trapnell, B. C., Carey, B. C., Uchida, K. & Suzuki, T. Pulmonary alveolar proteinosis, a primary immunodeficiency of impaired GM-CSF stimulation of macrophages. *Curr. Opin. Immunol.* **21,** 514–521 (2009).
450. Kaufmann, A. *et al.* Defense against influenza A virus infection: essential role of the chemokine system. *Immunobiology* **204,** 603–613 (2001).
451. Kaufmann, A. *et al.* Defense against influenza A virus infection: essential role of the chemokine system. *Immunobiology* **204,** 603–613 (2001).
452. Moltedo, B. *et al.* Cutting Edge: Stealth Influenza Virus Replication Precedes the Initiation of Adaptive Immunity. *J. Immunol.* **183,** 3569–3573 (2009).
453. Williams, A. E. *et al.* Innate imprinting by the modified heat-labile toxin of Escherichia coli (LTK63) provides generic protection against lung infectious disease. *J. Immunol. Baltim. Md 1950* **173,** 7435–7443 (2004).
454. Moyron-Quiroz, J. E. *et al.* Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nat. Med.* **10,** 927–934 (2004).
455. McGill, J., Van Rooijen, N. & Legge, K. L. Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs. *J. Exp. Med.* **205,** 1635–1646 (2008).
456. Hogan, R. J. *et al.* Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J. Immunol. Baltim. Md 1950* **166,** 1813–1822 (2001).
457. Hogan, R. J. *et al.* Protection from respiratory virus infections can be mediated by antigen-specific CD4(+) T cells that persist in the lungs. *J. Exp. Med.* **193,** 981–986 (2001).
458. Marshall, D. R. *et al.* Measuring the diaspora for virus-specific CD8+ T cells. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 6313–6318 (2001).
459. Ostler, T., Hussell, T., Surh, C. D., Openshaw, P. & Ehl, S. Long-term persistence and reactivation of T cell memory in the lung of mice infected with respiratory syncytial virus. *Eur. J. Immunol.* **31,** 2574–2582 (2001).
460. Wiley, J. A., Hogan, R. J., Woodland, D. L. & Harmsen, A. G. Antigen-specific CD8(+) T cells persist in the upper respiratory tract following influenza virus infection. *J. Immunol. Baltim. Md 1950* **167,** 3293–3299 (2001).
461. Ely, K. H. *et al.* Nonspecific recruitment of memory CD8+ T cells to the lung airways during respiratory virus infections. *J. Immunol. Baltim. Md 1950* **170,** 1423–1429 (2003).
462. Hogan, R. J. *et al.* Long-term maintenance of virus-specific effector memory CD8+ T cells in the lung airways depends on proliferation. *J. Immunol. Baltim. Md 1950* **169,** 4976–4981 (2002).

463. Christensen, J. P., Doherty, P. C., Branum, K. C. & Riberdy, J. M. Profound Protection against Respiratory Challenge with a Lethal H7N7 Influenza A Virus by Increasing the Magnitude of CD8+ T-Cell Memory. *J. Virol.* **74,** 11690–11696 (2000).
464. Lambe, T. *et al.* Immunity Against Heterosubtypic Influenza Virus Induced By Adenovirus And MVA Expressing Nucleoprotein And Matrix Protein-1. *Sci. Reports* **3,** 1443 (2013).
465. Jelley-Gibbs, D. M. *et al.* Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. *J. Exp. Med.* **202,** 697–706 (2005).
466. Kim, T. S., Hufford, M. M., Sun, J., Fu, Y.-X. & Braciale, T. J. Antigen persistence and the control of local T cell memory by migrant respiratory dendritic cells after acute virus infection. *J Exp Med* **207,** 1161–1172 (2010).
467. Zammit, D. J., Turner, D. L., Klonowski, K. D., Lefrançois, L. & Cauley, L. S. Residual Antigen Presentation after Influenza Virus Infection Affects CD8 T Cell Activation and Migration. *Immunity* **24,** 439–449 (2006).
468. Fesq, H., Bacher, M., Nain, M. & Gemsa, D. Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. *Immunobiology* **190,** 175–182 (1994).
469. Wilson, A. A. *et al.* Amelioration of emphysema in mice through lentiviral transduction of long-lived pulmonary alveolar macrophages. *J. Clin. Invest.* **120,** 379–389 (2010).
470. Zhao, Y. *et al.* High levels of virus-specific CD4+ T cells predict severe pandemic influenza A virus infection. *Am. J. Respir. Crit. Care Med.* **186,** 1292–1297 (2012).
471. Howe, S. J. *et al.* Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J. Clin. Invest.* **118,** 3143–3150 (2008).
472. Hacein-Bey-Abina, S. *et al.* Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J. Clin. Invest.* **118,** 3132–3142 (2008).
473. Cartier, N. *et al.* Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* **326,** 818–823 (2009).
474. Cavazzana-Calvo, M. *et al.* Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. *Nature* **467,** 318–322 (2010).
475. Philippe, S. *et al.* Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 17684–17689 (2006).
476. Apolonia, L. *et al.* Stable gene transfer to muscle using non-integrating lentiviral vectors. *Mol. Ther. J. Am. Soc. Gene Ther.* **15,** 1947–1954 (2007).
477. Negri, D. R. M. *et al.* Transduction of Human Antigen-Presenting Cells with Integrase-Defective Lentiviral Vector Enables Functional Expansion of Primed Antigen-Specific CD8+ T Cells. *Hum. Gene Ther.* **21,** 1029–1035 (2010).

478. Apolonia, L. *et al.* Stable gene transfer to muscle using non-integrating lentiviral vectors. *Mol. Ther. J. Am. Soc. Gene Ther.* **15,** 1947–1954 (2007).
479. Bayer, M. *et al.* A large U3 deletion causes increased in vivo expression from a nonintegrating lentiviral vector. *Mol. Ther. J. Am. Soc. Gene Ther.* **16,** 1968–1976 (2008).
480. Graham, M. B. *et al.* Response to influenza infection in mice with a targeted disruption in the interferon gamma gene. *J. Exp. Med.* **178,** 1725–1732 (1993).
481. Hussell, T., Pennycook, A. & Openshaw, P. J. Inhibition of tumor necrosis factor reduces the severity of virus-specific lung immunopathology. *Eur. J. Immunol.* **31,** 2566–2573 (2001).
482. Moskophidis, D. & Kioussis, D. Contribution of Virus-specific CD8+ Cytotoxic T Cells to Virus Clearance or Pathologic Manifestations of Influenza Virus Infection in a T Cell Receptor Transgenic Mouse Model. *J. Exp. Med.* **188,** 223–232 (1998).
483. Becker, T. C. *et al.* Interleukin 15 Is Required for Proliferative Renewal of Virus-specific Memory CD8 T Cells. *J. Exp. Med.* **195,** 1541–1548 (2002).
484. Schluns, K. S., Kieper, W. C., Jameson, S. C. & Lefrançois, L. Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. *Nat. Immunol.* **1,** 426–432 (2000).
485. Ku, C. C., Murakami, M., Sakamoto, A., Kappler, J. & Marrack, P. Control of Homeostasis of CD8+ Memory T Cells by Opposing Cytokines. *Science* **288,** 675–678 (2000).
486. Kunkel, E. J. *et al.* Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. *Am. J. Pathol.* **160,** 347–355 (2002).
487. Mora, J. R. *et al.* Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. *J. Exp. Med.* **201,** 303–316 (2005).
488. Campbell, D. J. & Butcher, E. C. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J. Exp. Med.* **195,** 135–141 (2002).
489. Agace, W. Generation of gut-homing T cells and their localization to the small intestinal mucosa. *Immunol. Lett.* **128,** 21–23 (2010).
490. Mora, J. R. *et al.* Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* **424,** 88–93 (2003).
491. Zammit, D. J., Turner, D. L., Klonowski, K. D., Lefrançois, L. & Cauley, L. S. Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. *Immunity* **24,** 439–449 (2006).
492. Ely, K. H., Cookenham, T., Roberts, A. D. & Woodland, D. L. Memory T cell populations in the lung airways are maintained by continual recruitment. *J. Immunol. Baltim. Md 1950* **176,** 537–543 (2006).
493. Richter, M. *et al.* Collagen distribution and expression of collagen-binding alpha1beta1 (VLA-1) and alpha2beta1 (VLA-2) integrins on CD4 and CD8 T cells during influenza infection. *J. Immunol. Baltim. Md 1950* **178,** 4506–4516 (2007).

494. Goodarzi, K., Goodarzi, M., Tager, A. M., Luster, A. D. & von Andrian, U. H. Leukotriene B4 and BLT1 control cytotoxic effector T cell recruitment to inflamed tissues. *Nat. Immunol.* **4,** 965–973 (2003).
495. Zhong, W. *et al.* Significant impact of sequence variations in the nucleoprotein on CD8 T cell-mediated cross-protection against influenza A virus infections. *PloS One* **5,** e10583 (2010).
496. Negri, D. R. M. *et al.* Successful immunization with a single injection of non-integrating lentiviral vector. *Mol. Ther. J. Am. Soc. Gene Ther.* **15,** 1716–1723 (2007).
497. Coutant, F., Frenkiel, M.-P., Despres, P. & Charneau, P. Protective antiviral immunity conferred by a nonintegrative lentiviral vector-based vaccine. *PloS One* **3,** e3973 (2008).
498. London, C. A., Lodge, M. P. & Abbas, A. K. Functional Responses and Costimulator Dependence of Memory CD4+ T Cells. *J. Immunol.* **164,** 265–272 (2000).
499. Croft, M., Bradley, L. M. & Swain, S. L. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J. Immunol.* **152,** 2675–2685 (1994).
500. Fuse, S., Zhang, W. & Usherwood, E. J. Control of Memory CD8+ T Cell Differentiation by CD80/CD86-CD28 Costimulation and Restoration by IL-2 during the Recall Response. *J. Immunol.* **180,** 1148–1157 (2008).
501. Bertram, E. M., Lau, P. & Watts, T. H. Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection. *J. Immunol. Baltim. Md 1950* **168,** 3777–3785 (2002).
502. Song, K. *et al.* Genetic immunization in the lung induces potent local and systemic immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 22213–22218 (2010).
503. Oakland, M., Sinn, P. L. & McCray Jr, P. B. Advances in Cell and Gene-based Therapies for Cystic Fibrosis Lung Disease. *Mol. Ther.* **20,** 1108–1115 (2012).
504. Broussau, S. *et al.* Inducible packaging cells for large-scale production of lentiviral vectors in serum-free suspension culture. *Mol. Ther. J. Am. Soc. Gene Ther.* **16,** 500–507 (2008).
505. Ikeda, Y. *et al.* Continuous high-titer HIV-1 vector production. *Nat. Biotechnol.* **21,** 569–572 (2003).
506. Witting, S. R. *et al.* Efficient Large Volume Lentiviral Vector Production Using Flow Electroporation. *Hum. Gene Ther.* **23,** 243–249 (2012).
507. Klages, N., Zufferey, R. & Trono, D. A stable system for the high-titer production of multiply attenuated lentiviral vectors. *Mol. Ther. J. Am. Soc. Gene Ther.* **2,** 170–176 (2000).
508. Ni, Y. *et al.* Generation of a packaging cell line for prolonged large-scale production of high-titer HIV-1-based lentiviral vector. *J. Gene Med.* **7,** 818–834 (2005).

509. Ahmad, A. *et al.* Kaposi sarcoma-associated herpesvirus-encoded viral FLICE inhibitory protein (vFLIP) K13 cooperates with Myc to promote lymphoma in mice. *Cancer Biol. Ther.* **10,** 1033–1040 (2010).
510. Chugh, P. *et al.* Constitutive NF-kappaB activation, normal Fas-induced apoptosis, and increased incidence of lymphoma in human herpes virus 8 K13 transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 12885–12890 (2005).
511. Andreakos, E., Williams, R. O., Wales, J., Foxwell, B. M. & Feldmann, M. Activation of NF-kappaB by the intracellular expression of NF-kappaB-inducing kinase acts as a powerful vaccine adjuvant. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 14459–14464 (2006).
512. Lenschow, D. J., Walunas, T. L. & Bluestone, J. A. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* **14,** 233–258 (1996).
513. Dempsey, P. W., Doyle, S. E., He, J. Q. & Cheng, G. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev.* **14,** 193–209 (2003).
514. Dempsey, P. W., Doyle, S. E., He, J. Q. & Cheng, G. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev.* **14,** 193–209 (2003).
515. Cannons, J. L., Choi, Y. & Watts, T. H. Role of TNF receptor-associated factor 2 and p38 mitogen-activated protein kinase activation during 4-1BB-dependent immune response. *J. Immunol. Baltim. Md 1950* **165,** 6193–6204 (2000).
516. Prell, R. A. *et al.* OX40-mediated memory T cell generation is TNF receptor-associated factor 2 dependent. *J. Immunol. Baltim. Md 1950* **171,** 5997–6005 (2003).
517. So, T., Salek-Ardakani, S., Nakano, H., Ware, C. F. & Croft, M. TNF receptor-associated factor 5 limits the induction of Th2 immune responses. *J. Immunol. Baltim. Md 1950* **172,** 4292–4297 (2004).
518. Nakano, H. *et al.* Targeted disruption of Traf5 gene causes defects in CD40- and CD27-mediated lymphocyte activation. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9803–9808 (1999).
519. Jang, I. K., Lee, Z. H., Kim, Y. J., Kim, S. H. & Kwon, B. S. Human 4-1BB (CD137) signals are mediated by TRAF2 and activate nuclear factor-kappa B. *Biochem Biophys Res Commun* **242,** 613 – 620 (1998).
520. Arch, R. H. & Thompson, C. B. 4-1BB and Ox40 Are Members of a Tumor Necrosis Factor (TNF)-Nerve Growth Factor Receptor Subfamily That Bind TNF Receptor-Associated Factors and Activate Nuclear Factor κB. *Mol. Cell. Biol.* **18,** 558–565 (1998).
521. Cannons, J. L., Hoeflich, K. P., Woodgett, J. R. & Watts, T. H. Role of the Stress Kinase Pathway in Signaling Via the T Cell Costimulatory Receptor 4-1BB. *J. Immunol.* **163,** 2990–2998 (1999).

522. Cannons, J. L., Choi, Y. & Watts, T. H. Role of TNF Receptor-Associated Factor 2 and p38 Mitogen-Activated Protein Kinase Activation During 4-1BB-Dependent Immune Response. *J. Immunol.* **165,** 6193–6204 (2000).
523. Arron, J. R., Pewzner-Jung, Y., Walsh, M. C., Kobayashi, T. & Choi, Y. Regulation of the subcellular localization of tumor necrosis factor receptor-associated factor (TRAF)2 by TRAF1 reveals mechanisms of TRAF2 signaling. *J. Exp. Med.* **196,** 923–934 (2002).
524. Sabbagh, L., Pulle, G., Liu, Y., Tsitsikov, E. N. & Watts, T. H. ERK-Dependent Bim Modulation Downstream of the 4-1BB-TRAF1 Signaling Axis Is a Critical Mediator of CD8 T Cell Survival In Vivo. *J. Immunol.* **180,** 8093–8101 (2008).
525. Nam, K.-O. *et al.* Cross-linking of 4-1BB activates TCR-signaling pathways in CD8+ T lymphocytes. *J. Immunol. Baltim. Md 1950* **174,** 1898–1905 (2005).
526. Taraban, V. Y. *et al.* Expression and costimulatory effects of the TNF receptor superfamily members CD134 (OX40) and CD137 (4-1BB), and their role in the generation of anti-tumor immune responses. *Eur. J. Immunol.* **32,** 3617–3627 (2002).
527. Roos, A., Schilder-Tol, E. J., Weening, J. J. & Aten, J. Strong expression of CD134 (OX40), a member of the TNF receptor family, in a T helper 2-type cytokine environment. *J. Leukoc. Biol.* **64,** 503–510 (1998).
528. Murata, K. *et al.* Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. *J. Exp. Med.* **191,** 365–374 (2000).
529. Miura, S. *et al.* Molecular cloning and characterization of a novel glycoprotein, gp34, that is specifically induced by the human T-cell leukemia virus type I transactivator p40tax. *Mol. Cell. Biol.* **11,** 1313–1325 (1991).
530. Ohshima, Y. *et al.* OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. *Blood* **92,** 3338–3345 (1998).
531. Imura, A. *et al.* The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. *J. Exp. Med.* **183,** 2185–2195 (1996).
532. Gramaglia, I., Weinberg, A. D., Lemon, M. & Croft, M. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J. Immunol. Baltim. Md 1950* **161,** 6510–6517 (1998).
533. Kawamata, S., Hori, T., Imura, A., Takaori-Kondo, A. & Uchiyama, T. Activation of OX40 signal transduction pathways leads to tumor necrosis factor receptor-associated factor (TRAF) 2- and TRAF5-mediated NF-kappaB activation. *J. Biol. Chem.* **273,** 5808–5814 (1998).
534. Rogers, P. R., Song, J., Gramaglia, I., Killeen, N. & Croft, M. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity* **15,** 445–455 (2001).
535. Gramaglia, I. *et al.* The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J. Immunol. Baltim. Md 1950* **165,** 3043–3050 (2000).

536. Bansal-Pakala, P., Halteman, B. S., Cheng, M. H.-Y. & Croft, M. Costimulation of CD8 T cell responses by OX40. *J. Immunol. Baltim. Md 1950* **172,** 4821–4825 (2004).
537. Lens, S. M., Tesselaar, K., van Oers, M. H. & van Lier, R. A. Control of lymphocyte function through CD27-CD70 interactions. *Semin. Immunol.* **10,** 491–499 (1998).
538. Bowman, M. R. *et al.* The cloning of CD70 and its identification as the ligand for CD27. *J. Immunol. Baltim. Md 1950* **152,** 1756–1761 (1994).
539. De Jong, R. *et al.* Regulation of expression of CD27, a T cell-specific member of a novel family of membrane receptors. *J. Immunol. Baltim. Md 1950* **146,** 2488–2494 (1991).
540. Akiba, H. *et al.* CD27, a member of the tumor necrosis factor receptor superfamily, activates NF-kappaB and stress-activated protein kinase/c-Jun N-terminal kinase via TRAF2, TRAF5, and NF-kappaB-inducing kinase. *J. Biol. Chem.* **273,** 13353–13358 (1998).
541. Gravestein, L. A. *et al.* The TNF receptor family member CD27 signals to Jun N-terminal kinase via Traf-2. *Eur. J. Immunol.* **28,** 2208–2216 (1998).
542. Lens, S. M., Tesselaar, K., van Oers, M. H. & van Lier, R. A. Control of lymphocyte function through CD27-CD70 interactions. *Semin. Immunol.* **10,** 491–499 (1998).
543. Harrop, J. A. *et al.* Herpesvirus entry mediator ligand (HVEM-L), a novel ligand for HVEM/TR2, stimulates proliferation of T cells and inhibits HT29 cell growth. *J. Biol. Chem.* **273,** 27548–27556 (1998).
544. Mauri, D. N. *et al.* LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity* **8,** 21–30 (1998).
545. Morel, Y. *et al.* Reciprocal Expression of the TNF Family Receptor Herpes Virus Entry Mediator and Its Ligand LIGHT on Activated T Cells: LIGHT Down-Regulates Its Own Receptor. *J. Immunol.* **165,** 4397–4404 (2000).
546. Tamada, K. *et al.* Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway. *Nat. Med.* **6,** 283–289 (2000).
547. Harrop, J. A. *et al.* Herpesvirus entry mediator ligand (HVEM-L), a novel ligand for HVEM/TR2, stimulates proliferation of T cells and inhibits HT29 cell growth. *J. Biol. Chem.* **273,** 27548–27556 (1998).
548. Horie, R. & Watanabe, T. CD30: expression and function in health and disease. *Semin. Immunol.* **10,** 457–470 (1998).
549. Wiley, S. R., Goodwin, R. G. & Smith, C. A. Reverse signaling via CD30 ligand. *J. Immunol. Baltim. Md 1950* **157,** 3635–3639 (1996).
550. Younes, A. *et al.* CD30 ligand is expressed on resting normal and malignant human B lymphocytes. *Br. J. Haematol.* **93,** 569–571 (1996).
551. Buchan, S. L. & Al-Shamkhani, A. Distinct Motifs in the Intracellular Domain of Human CD30 Differentially Activate Canonical and Alternative Transcription Factor NF-κB Signaling. *PLoS One* **7,** (2012).

552. Dempsey, P. W., Doyle, S. E., He, J. Q. & Cheng, G. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev.* **14,** 193–209 (2003).
553. Harlin, H., Podack, E., Boothby, M. & Alegre, M.-L. TCR-independent CD30 signaling selectively induces IL-13 production via a TNF receptor-associated factor/p38 mitogen-activated protein kinase-dependent mechanism. *J. Immunol. Baltim. Md 1950* **169,** 2451–2459 (2002).
554. Nocentini, G. *et al.* A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6216–6221 (1997).
555. McHugh, R. S. *et al.* CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* **16,** 311–323 (2002).
556. Tone, M. *et al.* Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15059–15064 (2003).
557. Stephens, G. L. *et al.* Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4+CD25+ T cells. *J. Immunol. Baltim. Md 1950* **173,** 5008–5020 (2004).
558. Spinicelli, S. *et al.* GITR interacts with the pro-apoptotic protein Siva and induces apoptosis. *Cell Death Differ.* **9,** 1382–1384 (2002).
559. Kwon, B. *et al.* Identification of a novel activation-inducible protein of the tumor necrosis factor receptor superfamily and its ligand. *J. Biol. Chem.* **274,** 6056–6061 (1999).
560. Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y. & Sakaguchi, S. Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* **3,** 135–142 (2002).
561. Ronchetti, S. *et al.* GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations. *Eur. J. Immunol.* **34,** 613–622 (2004).
562. Ji, H. *et al.* Cutting edge: the natural ligand for glucocorticoid-induced TNF receptor-related protein abrogates regulatory T cell suppression. *J. Immunol. Baltim. Md 1950* **172,** 5823–5827 (2004).
563. Kim, J. D., Kim, C. H. & Kwon, B. S. Regulation of mouse 4-1BB expression: multiple promoter usages and a splice variant. *Mol. Cells* **31,** 141–149 (2011).
564. Wilcox, R. A. *et al.* Cutting edge: Expression of functional CD137 receptor by dendritic cells. *J. Immunol. Baltim. Md 1950* **168,** 4262–4267 (2002).
565. Schwarz, H., Valbracht, J., Tuckwell, J., von Kempis, J. & Lotz, M. ILA, the human 4-1BB homologue, is inducible in lymphoid and other cell lineages. *Blood* **85,** 1043–1052 (1995).
566. Heinisch, I. V., Bizer, C., Volgger, W. & Simon, H. U. Functional CD137 receptors are expressed by eosinophils from patients with IgE-mediated allergic responses

but not by eosinophils from patients with non-IgE-mediated eosinophilic disorders. *J. Allergy Clin. Immunol.* **108,** 21–28 (2001).

567. Futagawa, T. *et al.* Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells. *Int. Immunol.* **14,** 275–286 (2002).
568. Reali, C. *et al.* Expression of CD137 and its ligand in human neurons, astrocytes, and microglia: modulation by FGF-2. *J. Neurosci. Res.* **74,** 67–73 (2003).
569. Broll, K., Richter, G., Pauly, S., Hofstaedter, F. & Schwarz, H. CD137 expression in tumor vessel walls. High correlation with malignant tumors. *Am. J. Clin. Pathol.* **115,** 543–549 (2001).
570. Boussaud, V., Soler, P., Moreau, J., Goodwin, R. G. & Hance, A. J. Expression of three members of the TNF-R family of receptors (4-1BB, lymphotoxin-beta receptor, and Fas) in human lung. *Eur. Respir. J. Off. J. Eur. Soc. Clin. Respir. Physiol.* **12,** 926–931 (1998).
571. Kwon, B. S., Kozak, C. A., Kim, K. K. & Pickard, R. T. Genomic organization and chromosomal localization of the T-cell antigen 4-1BB. *J. Immunol.* **152,** 2256–2262 (1994).
572. Kim, J.-O., Kim, H. W., Baek, K.-M. & Kang, C.-Y. NF-κB and AP-1 regulate activation-dependent CD137 (4-1BB) expression in T cells. *FEBS Lett.* **541,** 163–170 (2003).
573. Cheng, J. *et al.* Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science* **263,** 1759–1762 (1994).
574. Cascino, I., Papoff, G., De Maria, R., Testi, R. & Ruberti, G. Fas/Apo-1 (CD95) receptor lacking the intracytoplasmic signaling domain protects tumor cells from Fas-mediated apoptosis. *J. Immunol. Baltim. Md 1950* **156,** 13–17 (1996).
575. Kim, J. D., Kim, C. H. & Kwon, B. S. Regulation of mouse 4-1BB expression: Multiple promoter usages and a splice variant. *Mol. Cells* **31,** 141–149 (2011).
576. Langstein, J., Michel, J. & Schwarz, H. CD137 Induces Proliferation and Endomitosis in Monocytes. *Blood* **94,** 3161–3168 (1999).
577. Langstein, J. & Schwarz, H. Identification of CD137 as a potent monocyte survival factor. *J. Leukoc. Biol.* **65,** 829–833 (1999).
578. Lippert, U. *et al.* CD137 ligand reverse signaling has multiple functions in human dendritic cells during an adaptive immune response. *Eur. J. Immunol.* **38,** 1024–1032 (2008).
579. Kim, Y.-J., Li, G. & Broxmeyer, H. E. 4-1BB Ligand Stimulation Enhances Myeloid Dendritic Cell Maturation from Human Umbilical Cord Blood CD34+ Progenitor Cells. *J. Hematotherapy Htmlent Glyphamp Asciiamp Stem Cell Res.* **11,** 895–903 (2002).
580. Watts, A. D. A casein kinase I motif present in the cytoplasmic domain of members of the tumour necrosis factor ligand family is implicated in `reverse signalling'. *EMBO J.* **18,** 2119–2126 (1999).

581. Kang, Y. J. *et al.* Cell surface 4-1BBL mediates sequential signaling pathways 'downstream' of TLR and is required for sustained TNF production in macrophages. *Nat. Immunol.* **8,** 601–609 (2007).
582. Futagawa, T. *et al.* Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells. *Int. Immunol.* **14,** 275–286 (2002).
583. Hurtado, J. C., Kim, Y. J. & Kwon, B. S. Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death. *J. Immunol. Baltim. Md 1950* **158,** 2600–2609 (1997).
584. Bertram, E. M., Lau, P. & Watts, T. H. Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection. *J. Immunol. Baltim. Md 1950* **168,** 3777–3785 (2002).
585. Bukczynski, J., Wen, T., Ellefsen, K., Gauldie, J. & Watts, T. H. Costimulatory ligand 4-1BBL (CD137L) as an efficient adjuvant for human antiviral cytotoxic T cell responses. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1291–1296 (2004).
586. Shuford, W. W. *et al.* 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J. Exp. Med.* **186,** 47–55 (1997).
587. Takahashi, C., Mittler, R. S. & Vella, A. T. Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal. *J. Immunol. Baltim. Md 1950* **162,** 5037–5040 (1999).
588. McHugh, R. S. *et al.* CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* **16,** 311–323 (2002).
589. Elpek, K. G. *et al.* Ex vivo expansion of CD4+CD25+FoxP3+ T regulatory cells based on synergy between IL-2 and 4-1BB signaling. *J. Immunol. Baltim. Md 1950* **179,** 7295–7304 (2007).
590. Taraban, V. Y. *et al.* Expression and costimulatory effects of the TNF receptor superfamily members CD134 (OX40) and CD137 (4-1BB), and their role in the generation of anti-tumor immune responses. *Eur. J. Immunol.* **32,** 3617–3627 (2002).
591. Bukczynski, J. *et al.* Enhancement of HIV-Specific CD8 T Cell Responses by Dual Costimulation with CD80 and CD137L. *J. Immunol.* **175,** 6378–6389 (2005).
592. Wang, C. *et al.* 4-1BBL induces TNF receptor-associated factor 1-dependent Bim modulation in human T cells and is a critical component in the costimulation-dependent rescue of functionally impaired HIV-specific CD8 T cells. *J. Immunol. Baltim. Md 1950* **179,** 8252–8263 (2007).
593. Lin, G. H. Y. *et al.* Endogenous 4-1BB ligand plays a critical role in protection from influenza-induced disease. *J. Immunol. Baltim. Md 1950* **182,** 934–947 (2009).
594. Tan, J. T. *et al.* Analysis of expression and function of the costimulatory molecule 4-1BB in alloimmune responses. *Transplantation* **70,** 175–183 (2000).
595. Lin, G. H. Y. *et al.* Endogenous 4-1BB ligand plays a critical role in protection from influenza-induced disease. *J. Immunol. Baltim. Md 1950* **182,** 934–947 (2009).

596. Hendriks, J. *et al.* During Viral Infection of the Respiratory Tract, CD27, 4-1BB, and OX40 Collectively Determine Formation of CD8+ Memory T Cells and Their Capacity for Secondary Expansion. *J. Immunol.* **175,** 1665–1676 (2005).
597. Schluns, K. S. & Lefrançois, L. Cytokine control of memory T-cell development and survival. *Nat. Rev. Immunol.* **3,** 269–279 (2003).
598. Chu, N. R., DeBenedette, M. A., Stiernholm, B. J., Barber, B. H. & Watts, T. H. Role of IL-12 and 4-1BB ligand in cytokine production by CD28+ and CD28- T cells. *J. Immunol. Baltim. Md 1950* **158,** 3081–3089 (1997).
599. DeBenedette, M. A. *et al.* Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J. Immunol. Baltim. Md 1950* **163,** 4833–4841 (1999).
600. Tan, J. T. *et al.* 4-1BB Costimulation Is Required for Protective Anti-Viral Immunity After Peptide Vaccination. *J. Immunol.* **164,** 2320–2325 (2000).
601. Bertram, E. M. *et al.* A switch in costimulation from CD28 to 4-1BB during primary versus secondary CD8 T cell response to influenza in vivo. *J. Immunol. Baltim. Md 1950* **172,** 981–988 (2004).
602. Halstead, E. S., Mueller, Y. M., Altman, J. D. & Katsikis, P. D. In vivo stimulation of CD137 broadens primary antiviral CD8+ T cell responses. *Nat. Immunol.* **3,** 536–541 (2002).
603. Dawicki, W. & Watts, T. H. Expression and function of 4-1BB during CD4 versus CD8 T cell responses in vivo. *Eur. J. Immunol.* **34,** 743–751 (2004).
604. Fuse, S., Bellfy, S., Yagita, H. & Usherwood, E. J. CD8+ T cell dysfunction and increase in murine gammaherpesvirus latent viral burden in the absence of 4-1BB ligand. *J. Immunol. Baltim. Md 1950* **178,** 5227–5236 (2007).
605. Wang, C. *et al.* 4-1BBL induces TNF receptor-associated factor 1-dependent Bim modulation in human T cells and is a critical component in the costimulation-dependent rescue of functionally impaired HIV-specific CD8 T cells. *J. Immunol. Baltim. Md 1950* **179,** 8252–8263 (2007).
606. Pulle, G., Vidric, M. & Watts, T. H. IL-15-dependent induction of 4-1BB promotes antigen-independent CD8 memory T cell survival. *J. Immunol. Baltim. Md 1950* **176,** 2739–2748 (2006).
607. Jiang, D., Yue, P. S. E., Drenkard, D. & Schwarz, H. Induction of proliferation and monocytic differentiation of human CD34+ cells by CD137 ligand signaling. *Stem Cells Dayt. Ohio* **26,** 2372–2381 (2008).
608. Melero, I. *et al.* Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat. Med.* **3,** 682–685 (1997).
609. Miller, R. E. *et al.* 4-1BB-Specific Monoclonal Antibody Promotes the Generation of Tumor-Specific Immune Responses by Direct Activation of CD8 T Cells in a CD40-Dependent Manner. *J. Immunol.* **169,** 1792–1800 (2002).
610. Gramaglia, I., Cooper, D., Miner, K. T., Kwon, B. S. & Croft, M. Co-stimulation of antigen-specific CD4 T cells by 4-1BB ligand. *Eur. J. Immunol.* **30,** 392–402 (2000).

611. Guinn, B. A., DeBenedette, M. A., Watts, T. H. & Berinstein, N. L. 4-1BBL cooperates with B7-1 and B7-2 in converting a B cell lymphoma cell line into a long-lasting antitumor vaccine. *J. Immunol. Baltim. Md 1950* **162,** 5003–5010 (1999).
612. Harrison, J. M. *et al.* 4-1BBL coexpression enhances HIV-specific CD8 T cell memory in a poxvirus prime-boost vaccine. *Vaccine* **24,** 6867–6874 (2006).
613. Ganguly, S., Liu, J., Pillai, V. B., Mittler, R. S. & Amara, R. R. Adjuvantive effects of anti-4-1BB agonist Ab and 4-1BBL DNA for a HIV-1 Gag DNA vaccine: different effects on cellular and humoral immunity. *Vaccine* **28,** 1300–1309 (2010).
614. Kanagavelu, S. K. *et al.* Soluble multi-trimeric TNF superfamily ligand adjuvants enhance immune responses to a HIV-1 Gag DNA vaccine. *Vaccine* **30,** 691–702 (2012).
615. Du, X. *et al.* The adjuvant effects of co-stimulatory molecules on cellular and memory responses to HBsAg DNA vaccination. *J. Gene Med.* **9,** 136–146 (2007).
616. Moraes, T. J., Lin, G. H. Y., Wen, T. & Watts, T. H. Incorporation of 4-1BB ligand into an adenovirus vaccine vector increases the number of functional antigen-specific CD8 T cells and enhances the duration of protection against influenza-induced respiratory disease. *Vaccine* **29,** 6301–6312 (2011).
617. Bukczynski, J., Wen, T., Ellefsen, K., Gauldie, J. & Watts, T. H. Costimulatory ligand 4-1BBL (CD137L) as an efficient adjuvant for human antiviral cytotoxic T cell responses. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1291–1296 (2004).
618. Suhoski, M. M. *et al.* Engineering artificial antigen-presenting cells to express a diverse array of co-stimulatory molecules. *Mol. Ther. J. Am. Soc. Gene Ther.* **15,** 981–988 (2007).
619. Zhang, G. Tumor necrosis factor family ligand-receptor binding. *Curr. Opin. Struct. Biol.* **14,** 154–160 (2004).
620. Li, Q., Carr, A., Ito, F., Teitz-Tennenbaum, S. & Chang, A. E. Polarization effects of 4-1BB during CD28 costimulation in generating tumor-reactive T cells for cancer immunotherapy. *Cancer Res.* **63,** 2546–2552 (2003).
621. Wilcox, R. A. *et al.* Cutting edge: Expression of functional CD137 receptor by dendritic cells. *J. Immunol. Baltim. Md 1950* **168,** 4262–4267 (2002).
622. Laderach, D., Wesa, A. & Galy, A. 4-1BB-ligand is regulated on human dendritic cells and induces the production of IL-12. *Cell. Immunol.* **226,** 37–44 (2003).
623. Kuang, Y. *et al.* Effects of 4-1BB signaling on the biological function of murine dendritic cells. *Oncol. Lett.* **3,** 477–481 (2012).
624. Choi, B. K. *et al.* 4-1BB functions as a survival factor in dendritic cells. *J. Immunol. Baltim. Md 1950* **182,** 4107–4115 (2009).
625. Haan, J. M. M. den, Lehar, S. M. & Bevan, M. J. Cd8+ but Not Cd8− Dendritic Cells Cross-Prime Cytotoxic T Cells in Vivo. *J. Exp. Med.* **192,** 1685–1696 (2000).
626. Henri, S. *et al.* The dendritic cell populations of mouse lymph nodes. *J. Immunol. Baltim. Md 1950* **167,** 741–748 (2001).

627. Vremec, D., Pooley, J., Hochrein, H., Wu, L. & Shortman, K. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J. Immunol. Baltim. Md 1950* **164,** 2978–2986 (2000).
628. Heath, W. R. & Villadangos, J. A. No driving without a license. *Nat. Immunol.* **6,** 125–126 (2005).
629. Futagawa, T. *et al.* Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells. *Int. Immunol.* **14,** 275–286 (2002).
630. Niu, L. *et al.* Cytokine-Mediated Disruption of Lymphocyte Trafficking, Hemopoiesis, and Induction of Lymphopenia, Anemia, and Thrombocytopenia in Anti-CD137-Treated Mice. *J. Immunol.* **178,** 4194–4213 (2007).
631. Lin, G. H. Y., Snell, L. M., Wortzman, M. E., Clouthier, D. L. & Watts, T. H. GITR-Dependent Regulation of 4-1BB Expression: Implications for T Cell Memory and Anti–4-1BB–Induced Pathology. *J. Immunol.* **190,** 4627–4639 (2013).
632. Wang, J. *et al.* CD137-Mediated Pathogenesis from Chronic Hepatitis to Hepatocellular Carcinoma in Hepatitis B Virus-Transgenic Mice. *J. Immunol.* **185,** 7654–7662 (2010).
633. Ascierto, P. A., Simeone, E., Sznol, M., Fu, Y.-X. & Melero, I. Clinical Experiences With Anti-CD137 and Anti-PD1 Therapeutic Antibodies. *Semin. Oncol.* **37,** 508–516 (2010).